A STUDY OF
OLFACTORY MECHANISMS

by

DAVID JAMES SQUIRRELL, BSc.

A thesis submitted for the degree of Doctor of Philosophy

in the

Department of Chemistry and Molecular Science

at the

University of Warwick

December, 1978

CONTENTS

ABBREVIATIONS

cyclic AMP	Adenosine-3',5'-cyclic monophosphoric acid
8-bromo cyclic AMP	8-bromo adenosine-3',5'-cyclic monophosphoric acid
dibutyryl cyclic AMP	N^6, $O^{2'}$-dibutyryl adenosine-3',5'-cyclic monophosphoric acid
Na^+,K^+-ATPase	sodium-potassium adenosine triphosphatase
D.E.G.S.	diethyleneglycolsuccinate
EOG	electro-olfactogram
cyclic GMP	guanosine-3',5'-cyclic monophosphoric acid
8-bromo cyclic GMP	8-bromo guanosine-3',5'-cyclic monophosphoric acid
dibutyryl cyclic GMP	N^2,$O^{2'}$-dibutyryl guanosine-3',5'-cyclic monophosphoric acid
i.d.	inside diameter
p.s.i.	pounds per square inch
P.T.F.E., (Teflon)	polytetrafluorethene
SQ 20,009	1-ethyl-4(isopropylidenehydrazine)-1H-pyrazole-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, hydrochloride
v/v	volume/volume
w/v	weight/volume

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. G.H. Dodd for his help and guidance throughout the course of this work.

I would also like to thank my colleagues, Adnan, Francis, Julia, Krishna and Mike for useful discussion. I am indebted to Michael Poynder for the loan of various items of equipment and helpful suggestions; to Malcolm Farmer and Steve Speed for synthesising some of the compounds for me; and to Krishna Persaud for the gas sensor used for monitoring vapour phase odorant concentrations.

Bush Boake Allen Ltd., London, E15, provided financial support for this work through a C.A.S.E. award for which I am grateful.

Finally, I would like to mention Liz for the typing and the encouragement she has given me.

SUMMARY

A structure-activity relationship study examining the change in odour quality with systematic variation of alkyl and alkanoate groups of esters (up to C_{10}) has shown that an exposed ester group is necessary for giving esters a fruity smell. Steric hindrance of the ester group results in a minty odour quality becoming predominant.

Alkyl haloacetates have been shown to be capable of specifically inhibiting EOG responses in a manner most easily explained by an affinity labelling mechanism. Such compounds may be useful as a means of labelling olfactory receptors so that they may be identified during isolation.

Experiments to clarify the role of cyclic AMP in the olfactory transduction mechanism showed that odorant in solution (pentyl acetate) was antagonistic to the effects of SQ 20,009, a potent phosphodiesterase inhibitor. Positive after-potentials to ethanol were stimulated by SQ 20,009, suggesting an alternative mechanism for the effects of cyclic AMP on the EOG response involving increased permeability of the supporting cell membrane to chloride ions.

Biochemical studies in olfaction are hampered by the lack of a method for isolating the chemoreceptive membrane. Here experiments have been carried out at the level of the whole tissue using the EOG response to monitor events in the receptor cells.

EOG responses, stable over a period of several hours, were obtained from mammalian olfactory epithelium maintained *in vitro*. Animals such as the sheep and the cow can provide large quantities of tissue that may be necessary for biochemical studies. The preparation described here provides a convenient method of obtaining electrophysiological responses from the olfactory epithelium of such animals.

CHAPTER 1 INTRODUCTION - THE VERTEBRATE OLFACTORY SYSTEM

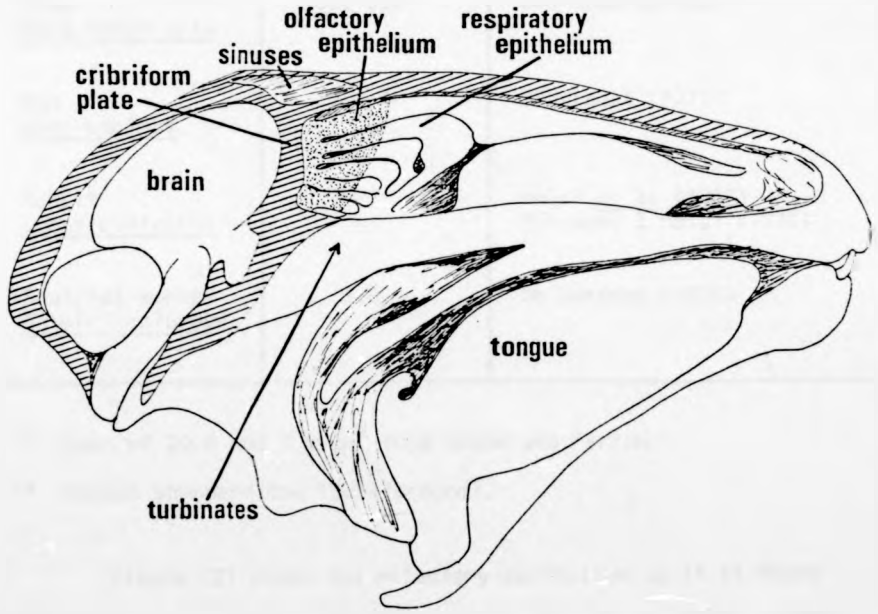
1.1 Morphology

1.1 i) Macroscopic

The sensory part of the olfactory system in the vertebrates takes the form of a pseudo-stratified columnar epithelium. It is found as a discrete portion of the tissue lining the nasal passages and may be readily distinguished by its yellowish pigmentation. There is a sharp delineation between the sensory tissue - the olfactory epithelium - and the non-sensory respiratory epithelium which lines the remainder of the surface of the nasal cavity.

Figure (1) overleaf is a diagram of a sagittally bisected sheep's head with the various features labelled. The olfactory epithelium can be seen to lie upon a series of convoluted bones, called turbinates and on the cribriform plate. It is also found on the posterior part of the septum which has been dissected away to reveal the nasal cavity. The turbinates are rigid to hold opposing faces of the epithelium apart and the system allows a large area of exposed sensory tissue to be packed into a small space. The same basic structure is found throughout the Mammalia with the number of turbinates and the area of olfactory epithelium varying, (see Table (1) below). Descriptions of the comparative morphology may be found in Parsons, (1971), and Allison, (1953), whilst Heist et al, (1967), working on the rabbit, have defined the morphology and the localisation of the olfactory epithelium.

FIGURE (1)



BISECTED SHEEP HEAD WITH NASAL SEPTUM REMOVED

TABLE (1) Comparison of total areas of olfactory epithelium in several species.

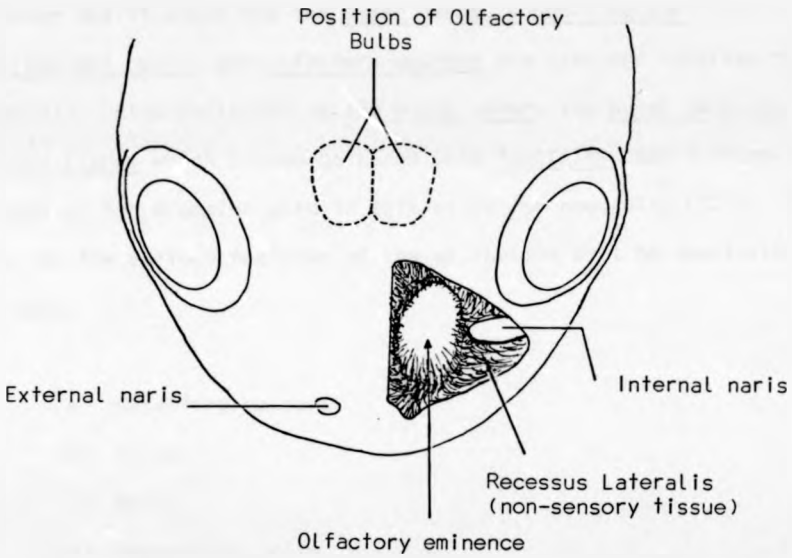
Species	Total Area Of Olfactory Epithelium cm^2	Reference
Cat *Felis domesticus*	13.9*	Negus (1958) Ferron (1973)
Dog** *Canis familiaris*	150	Müller (1955)
Frog *Rana temporaria*	*ca.* 0.5	own observations
Man *Homo sapiens*	2 - 4	Graziadei (1971)
Rabbit *Lepus cuniculus*	7.27	Heist *et al* (1967) Mulvaney & Heist (1970)
Squirrel monkey *Saimiri sciureus*	3.0	De Lorenzo (1970)

* Mean of 20.8 and 7.0 cm^2 from Negus and Ferron

** German shepherd dog (Schäferhund).

Figure (2) shows the olfactory epithelium as it is found in the frog *Rana temporaria* which is typical for the Amphibia. The structure is simpler than that in mammals and there is no sharp visual delineation between olfactory and respiratory epithelium. The diagram shows the ventral part of the nasal cavity, but sensory tissue also lines the dorsal part.

FIGURE 2



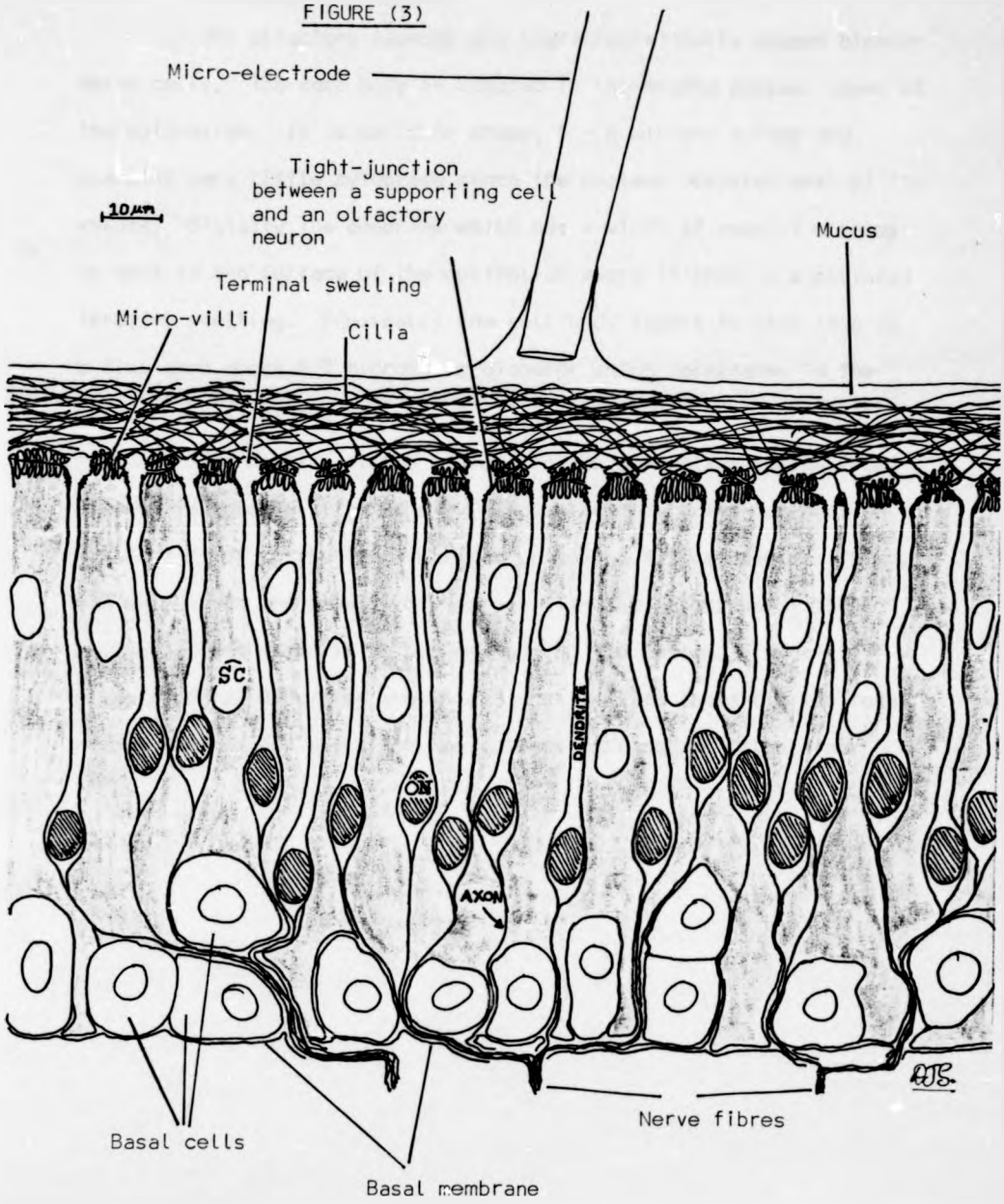
Frog's head after dissection to expose nasal cavity, showing position of olfactory bulbs. The dorsal olfactory epithelium has been removed.

1.1 ii) Microscopic

The cell structure of the olfactory epithelium as observed by light and electron microscopy follows a relatively constant pattern in all the vertebrates so far studied. There are three cell types present with their nuclei arranged in slightly overlapping layers. Figure (3) is a scale diagram of a generalised olfactory epithelium and it shows how the basal cells, supporting (or sustentacular) cells, and olfactory neurons are disposed relative to one another. Also indicated is the mucus layer, the basal membrane, the nerve fibres which become gathered into fascicles, and a micro-electrode of the diameter used in this study for recording EOG's. The details of the various features of the epithelium will be dealt with as follows:

a) Olfactory neurons
b) Cilia
c) Mucus
d) Supporting cells
e) Basal cells
f) Axons
g) Contacts between cells
h) The lamina propria

FIGURE (3)



ON - Olfactory neuron

SC - Supporting cell

Scale: 1 millimetre = 1 micron

Scale drawing of a generalised vertebrate olfactory epithelium.

a) Olfactory neurons

The olfactory neurons are characteristically shaped bipolar nerve cells. The cell body is located in the middle nuclear layer of the epithelium. It is ovoid in shape, 5 - 8 microns across and contains very little cytoplasm since the nucleus occupies most of its volume. Distally the dendrite which has a width of about 2 microns is sent to the surface of the epithelium where it ends in a ciliated terminal swelling. Proximally the cell body tapers to give rise to a fine axon about 0.2 microns in diameter which terminates in the olfactory bulb of the brain.

The density of the neurons in the epithelium varies from species to species. The density also varies according to location within a given epithelium. Working on bovine tissue, Menco, (1977), has correlated the density of neurons with the thickness of the epithelium and shown it to be greatest on the septum. Table (II) gives neuronal densities and in conjunction with Table (I) the total number of receptor cells for the animals included is calculated.

TABLE (11) Comparison of the number of olfactory neurons in several species.

Species	Total Area of Olfactory Epithelium (cm^2) *	Density of Olfactory Neurons per $cm^2 \times 10^{-6}$	References	Calculated No. of Olfactory Neurons (millions)
Cat	13.9	9.6	Ferron (1973)	133
Dog	150	1.5	" Muller (1955)	225
Frog	0.5	1.5	Menco (1977)	0.75
Man	2-4	3.0	Kanda et al (1973)	9.0
Rabbit	7.27	9.0	Heist et al (1967)	65.5
Squirrel monkey	3.0	-	-	-

* From TABLE (1)

b) Cilia

The olfactory neurons bear cilia which arise from the terminal swelling on the apical end of the dendrite. This terminal swelling protudes slightly above the level of the surrounding supporting cells.

The proximal portion of the cilia has a diameter of about 0.25 microns and a normal 9(2)+2 axonemal structure, but after a few microns there is a distinct narrowing of the cilium to a diameter of about 0.15 microns and from here on the cilium gradually tapers to about 0.06 microns in diameter. The gradual tapering of the distal part of the cilium is accompanied by a progressive reduction in the number of microtubules thus any cross-section through the mucus shows cilia with varying numbers of microtubules according to the point at which each has been cut. (See review by Steinbrecht, 1969).

Both the number of cilia per neuron and the length of the cilia varies from species to species. Estimates of the number per neuron stretch from 1-6 for the mole, (Graziadei, 1966), to 100-150 for the dog, (Okano, 1967). For the animals used in this study, frog and sheep, the respective values are 6-8, (Reese, 1965), and 40-50, (Kratzing, 1970). The determination of ciliary length is difficult: the thin sections observed microscopically are unlikely to contain the full length of any cilium and the fragility of the cilia renders them liable to breakage during fixation. Lengths are thus likely to be underestimated. The largest figure so far reported is by Reese, (1965), using light microscopy who gives 80-200 microns for the length of frog olfactory cilia.

Olfactory cilia are unlike the respiratory cilia of the surrounding tissue which maintain a constant diameter and retain a 9(2)+2 structure of microtubules throughout their length. The respiratory cilia beat in a synchronous wave-like pattern whereas any motion that the olfactory cilia display is unco-ordinated and irregular.

Menco et al, (1976), using freeze-fracture techniques for electron microscopy have demonstrated the presence of large numbers of particles in the membranes of the olfactory cilia compared to corresponding respiratory cilia in bovines. It is postulated that these particles might represent olfactory receptor sites. Menco, (1978), has subsequently found similar particles in the olfactory cilia of rat, dog and frog, and Usukura and Yamada, (1978), have found them in newt olfactory cilia.

c) Mucus

A layer of mucus is found overlying the olfactory receptors in all classes of vertebrates. The mucus layer is thicker and more concentrated in the frog than in mammals, (Heist et al, 1967). In amphibians and reptiles the mucus is secreted partly by the supporting cells and partly by the sub-epithelial Bowman's Glands, whilst in mammals and birds the only source of mucus is the Bowman's Gland, (Bannister, 1974).

The mucus is mobile at the surface. Charcoal dust sprinkled on the surface of frog olfactory epithelium can be seen to be removed by flowing mucus, (Reese, 1965 and own observations). The chemical composition of the mucus is poorly known. In man the nasal mucus is over 95% water with salts, mucin, muraminidase and

secretory immunglobulin present, (Abramson and Harker, 1973). It has a pH of about 7.0. The air/mucus partition coefficient for odorants and their diffusion rates through the mucus will control access of odorant molecules to the receptors, (Bostock, 1974,a).

d) Supporting cells

The supporting cells have a larger cell volume than the neurons, which they surround at the level of the dendrites. Theirs is the upper band of nuclei in the epithelium. The distal parts of the supporting cells approximate to being cylindrical in shape whilst they are compressed at the level of the neuronal cell bodies. Cellular feet are sent down past the basal cells to contact the basal membrane. The apical surface of the supporting cells is rounded and has microvilli which vary in density and dimensions according to species.

Electron micrographs of frog supporting cells consistently show them to be packed with large secretory droplets (e.g. Yamamoto et al, 1965), and the same is true for the newt (e.g. Usukura and Yamada, 1978). This feature is not found in mammals or birds.

The ratio of supporting cells to neurons is approximately 1:1, (Alcock, 1901, Heist et al, 1967).

e) Basal cells

The layer of nuclei deepest in the olfactory epithelium belongs to the basal cells. The cells lie adjacent to the basal membrane and amongst the proximal processes of the supporting cells. They ensheath groups of axons.

f) Axons

The mean diameter of the axons is 0.2 microns, (Graziadei, 1971). They stretch from the cell bodies of the olfactory neurons to the glomeruli of the olfactory bulb without synapsing. Between the cell bodies and the basal membrane they are grouped into small bundles and leave the basal membrane to be ensheathed by Schwann cells 15-40 at a time, (Gasser, 1956). They then turn to run parallel to the basal membrane being grouped into progressively larger bundles before turning at right angles to assume a vertical course through the cribriform plate to the brain. Individual fibres are not separated by Schwann cells.

Allison and Warwick, (1949), estimate that in the rabbit each glomerulus is, on average, connected to 26,000 axons.

g) Contacts between cells

Sectioning the olfactory epithelium parallel to the surface of the cell layer shows the terminal swellings of the dendrites to be spaced apart by the supporting cells which have a larger cross-sectional area. At this level cells are connected to one another by a tight-junctional belt. Reese and Brightman, (1970), have shown that intravenously injected horseradish peroxidase cannot diffuse past these junctions to the epithelial surface. Thus the tight junctions prevent diffusion between the mucus and the inter-cellular spaces within the epithelium. They may also serve to give the epithelium mechanical strength.

Contacts between the neurons occur only occassionally at the level of the dendrites and terminal swellings, but the cell bodies frequently touch one another. As mentioned above, the axons are in

close contact with each other.

h) The lamina propria

The lamina propria separates the olfactory epithelium from the underlying bone. It consists of connective tissue, olfactory nerve fibres, a vascular system and the mucus-secreting Bowman's Glands, the ducts of which extend to the epithelial surface.

1.1 iii) Cell dynamics in the olfactory epithelium

Despite the generally held belief that nerve cells in adult vertebrates are unable to be regenerated, there is a continuous process of cell renewal in the olfactory epithelium, with the basal cells acting as stem cells. This renewal of cells may be made essential because the olfactory epithelium is exposed to the external environment and may undergo continual attrition.

A turnover of cells has been suggested by morphological studies. For example, Thornhill, (1967), investigating the olfactory epithelium of the lamprey, found that there was always a proportion of degenerating neurons and supporting cells, but that there were no degenerating basal cells.

Moulton et al, (1970), working on mice, Graziadei and Metcalf, (1971), working on frogs, and Moulton and Fink, (1972), studying both, used autoradiographical techniques to follow cell movements in the epithelium. By injecting tritiated thymidine, cells that had undergone mitosis were labelled and could be traced.

Their results showed that the basal cells divide either to give more basal cells or to undergo differentiation into olfactory neurons and supporting cells. Such differentiating cells were found to migrate towards the surface of the epithelium whilst new basal cells remained adjacent to the basal membrane. Graziadei and Metcalf also observed the division of supporting cells.

Graziadei, (1973), found that in the frog the rate of turnover of neurons is greater than the rate of turnover of supporting cells. Moulton, (1975), used colchicine to block cells in metaphase in mouse olfactory epithelium and estimated that the olfactory neurons have a turnover time of about 29 days.

1.1 iv) Experimentally induced degeneration and subsequent regeneration of the olfactory epithelium

Concomitant with the ability of the olfactory epithelium to undergo a continuous process of cell renewal is its ability to regenerate following experimental destruction of the nerve cell population by treatments such as sectioning the olfactory nerve, ablating the olfactory bulb or exposing the epithelium to zinc sulphate solutions.

The literature on this subject up until 1971 has been reviewed by Takagi, who concluded that it was "highly conceivable" that new olfactory neurons could be generated from undifferentiated stem cells to enable the tissue to recover from insult. Subsequent work seems to have proved the point.

Regeneration after nerve sectioning has been demonstrated in the frog by Graziadei, (1973) and in the pigeon by Tucker et al, (1975) and Bedini et al, (1976). Regeneration of the mouse epithelium has been shown after zinc sulphate treatment (Matulionis, 1975) and after complete removal of the olfactory bulb, (Graziadei, 1978).

Nerve sectioning or olfactory bulb ablation causes retrograde degeneration of the axons and all the neurons in the epithelium within a few days. This leaves just supporting cells and basal cells, but the latter are stimulated by the degradation of the neurons into intense activity. The basal cells divide and differentiate and within a few weeks the epithelium is reconstituted and the axons have grown back to make connections with the glomeruli of the olfactory bulb.

Matulionis' treatment of mouse olfactory epithelium with a 1% aqueous zinc sulphate solution also caused degeneration of the supporting cells. These too were able to be replaced.

Finally, in the experiments by Tucker et al, (1975), on pigeons it was shown that the degeneration and subsequent regeneration of the olfactory neurons, which was followed morphologically, was accompanied by behavioural and electrophysiological changes that paralleled the changes in cell structure.

1.2 Electrical Activity In The Olfactory Epithelium

The interaction of the olfactory epithelium with odorous stimuli is signalled by electrical activity from the olfactory neurons. Their response at various levels may be examined by use of the appropriate recording techniques.

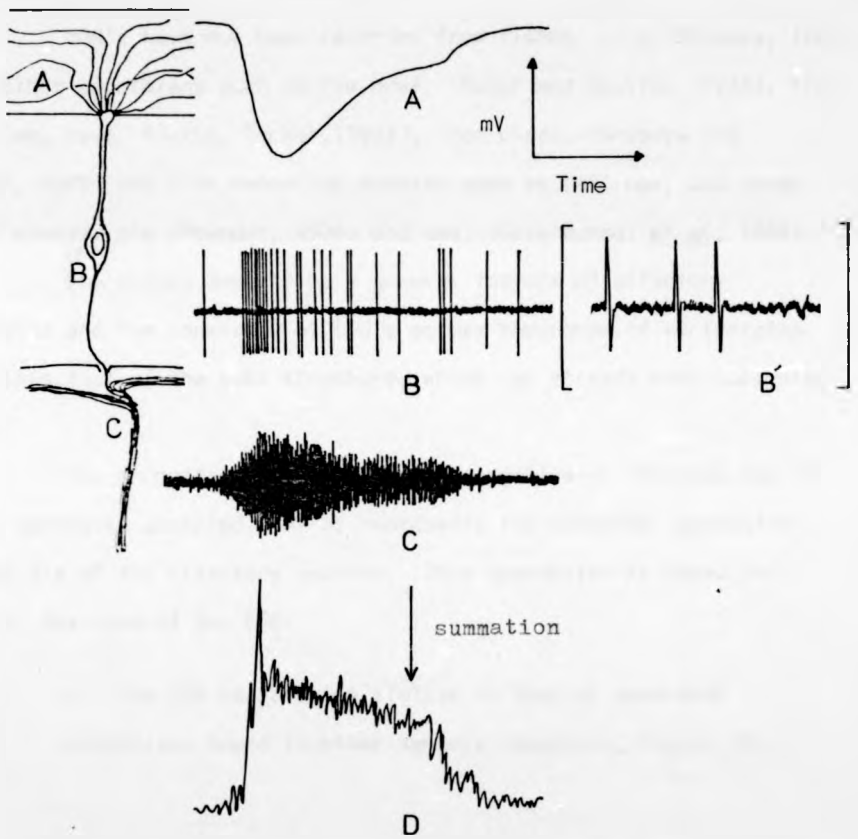
An electrode placed in the mucus at the surface of the epithelium enables the summated generator potentials of the neurons to be recorded. What is observed is a slow, sustained potential change of up to several millivolts in amplitude. This is called the electro-olfactogram, or EOG, a term coined by Ottoson, (1956), in analogy to the ERG from the retina.

A fine micro-electrode inserted into the epithelium in the region of the cell bodies or axons permits the action potentials from individual neurons to be monitored, whilst recording from the olfactory nerve, or bundles or axons in it, allows the impulse activity from a large number of neurons to be observed, and this activity may be integrated to give a summation of the neuronal discharges.

Figure (4) has been drawn to give an idea of what the outputs from these recordings look like and so that they may be directly compared since they will subsequently be treated separately.

FIGURE (4)

A diagrammatic representation of nerve activities recorded from different levels in the epithelium.



A Electro-olfactogram from Gesteland et al, (1965)

B Excitatory single-cell impulse activity based on van Drongelen, (1978)

B' Impulse activity on an expanded time scale from Gesteland et al, (1963)

C Impulse activity in the olfactory nerve based on Shibuya, (1964)

D Summated neural discharge based on Tucker and Shibuya, (1965)

The olfactory neuron on the left is drawn to show the positions from which the responses are obtained.

1.2 i) The electro-olfactogram or EOG

EOG's were first recorded by Hosoya and Yoshida, (1937), from canine olfactory epithelium. Subsequently, Ottoson, having observed EOG's in the rabbit, (Ottoson, 1954), made a detailed study of the phenomenon in the frog, (Ottoson, 1956).

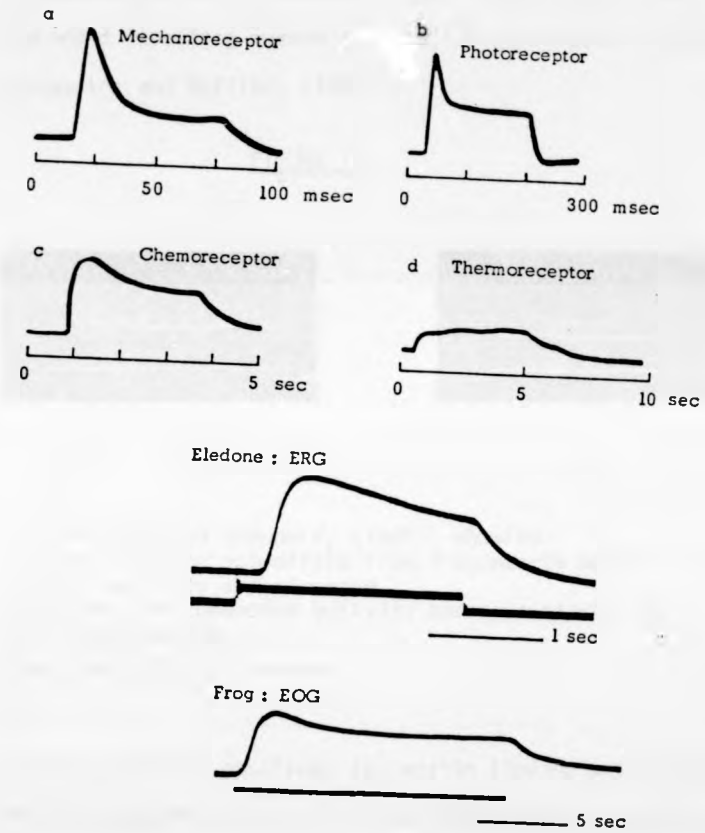
EOG's have now been recorded from fishes, (e.g. Shibuya, 1960), from other amphibians such as the newt, (Kauer and Moulton, 1974), from reptiles, (e.g. turtle, Tucker,(1963), from birds, (Shibuya and Tucker, 1965) and from mammalian species such as rat, cow, and sheep (this study), pig (Poynder, 1978) and man, (Osterhammel et al, 1969).

EOG's thus seem to be a general feature of olfactory epithelia and the constancy of EOG's across the range of vertebrates parallels that of the cell structure, which has already been commented upon.

The properties exhibited by the negative-on EOG have led it to be generally accepted that it represents the summated generator potentials of the olfactory neurons. This assumption is based on several features of the EOG:

a) The EOG has a shape similar to that of generator potentials found in other sensory receptors, Figure (5).

FIGURE (5)



A comparison of receptor potentials from:
a) frog muscle spindle
b) blowfly retinula cell
c) antenna of male cockroach
d) infra-red receptor
e) eye of Eledone
f) from the olfactory organ of frog.

From Ottoson, (1974)

See also Fuortes, (1971)

b) The EOG is not abolished by cocaine at a concentration sufficient to block the impulse activity of the olfactory nerve (Ottoson, 1956, Kimura, 1961). This is a general feature of generator potentials such as that recorded from frog muscle spindle, (Figure (6)). See also Eyzaguire and Kuffler, (1955).

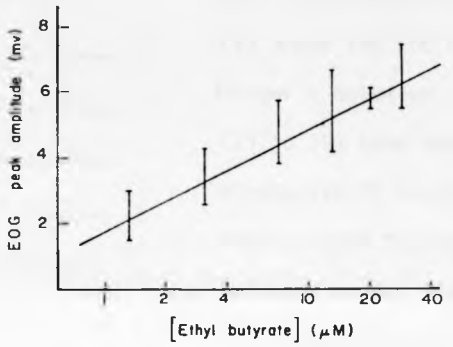
FIGURE (6)

(a) (b)

From Ottoson and Shepherd, (1965), showing:
(a) the receptor potentials from frog muscle spindle with impulse activity superimposed
(b) after the conducted activity has been blocked by 0.2% lignococaine
Time bar: 50 milli-seconds

c) The EOG peak amplitude is, within limits, proportional to the logarithm of the stimulus concentration - it is a stimulus-related, graded response. See Figure (7) from Gesteland and Sigwart, (1977), and also Ottoson, (1956), and Poynder, (1974,a,b).

FIGURE (7)

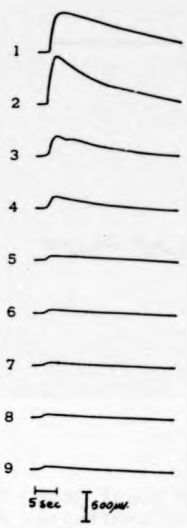


Variation in peak amplitude of EOG's from rat versus log concentration of odorant, ethyl butyrate, (micro-Moles per litre of air).
Vertical bars indicate range of values measured for 10 animals. From Gesteland and Sigwart, (1977).

d) Anti-dromic stimulation of the olfactory nerve does not result in potential changes at the mucus level, thus the EOG is not simply a product of the activity of the nerve fibres, (Ottoson, 1956, 1959).

e) The ionic basis for EOG generation has been studied by Takagi et al, (1966, 1968 and 1969) and shown to be mainly dependent upon Na^+ entry, see Figure (8). This mechanism is usual for depolarising generator potentials.

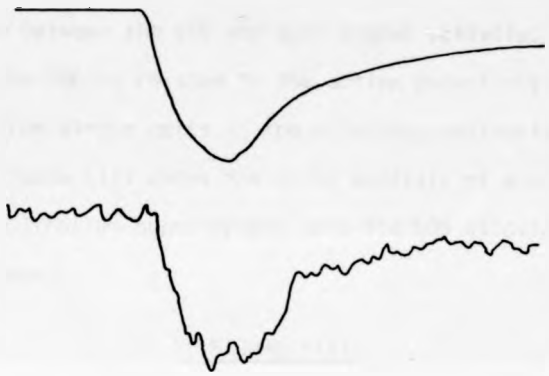
FIGURE (8)



EOG's to menthone vapour in Na^+ free Ringer's solution. (1) shows the EOG in normal Ringer's solution (2) to (9) show records taken at successive 20 minute intervals from excised frog epithelium immersed in Na^+ free solution. From Takagi et al, (1968).

f) Gesteland et al, (1965), have shown that the EOG is accompanied by a change in transepithelial impedance, (see Figure (9)). This is what would be expected since a change in membrane permeability is necessary for a generator current to flow.

FIGURE (9)

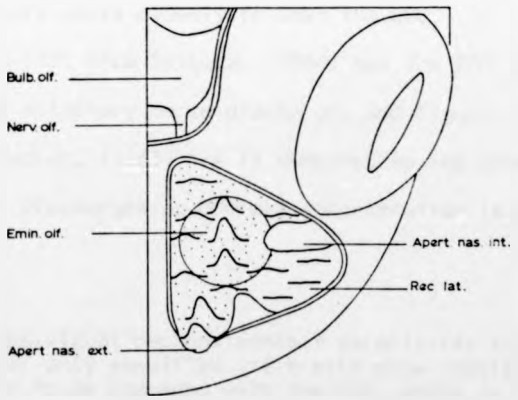


The upper trace shows the EOG whilst the lower trace shows the variations in transepithelial impedence during stimulation with odorant. (Sweep length 10 seconds). From Gesteland et al, (1965).

g) EOG's can only be obtained from sensory epithelium.

See Figure (10) from Ottoson, (1956).

FIGURE (10)

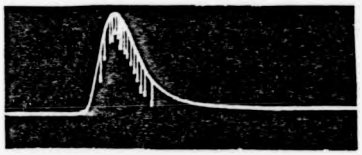


Schematic diagram showing responses to stimulation of restricted areas of frog olfactory mucosa. Dotted area indicates sensory epithelium. (From Ottoson, 1956).

h) A generator potential is the causal event for a chain of activity at higher levels, so a direct relationship must be shown between the EOG and such higher activity.

i) The EOG is related to the action potentials recorded from single cells in the olfactory epithelium.*
Figure (11) shows the spike activity of a unit in frog epithelium superimposed onto the EOG elicited by a musk.

FIGURE (11)



The EOG and single unit activity from frog.
From Gesteland et al, (1963).

ii) Kimura, (1961), showed that impulse activity in the olfactory nerve closely follows the EOG.
Figure (12) from Shibuya, (1964) has the EOG compared to the olfactory nerve discharge, and Figure (13) from Tucker, (1963) has it compared to the summated neural discharges as odorant concentration is varied.

* Since individual neurons exhibit selectivity towards odorants, only sensitive units will show impulse activity to be compared with the EOG, which is a mass response.

FIGURE (12)

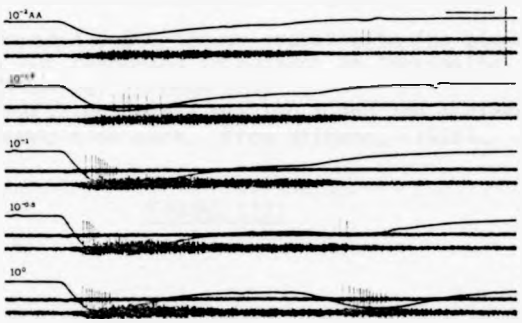
The EOG superimposed upon impulse activity in the olfactory nerve. From Shibuya, (1964).

FIGURE (13)

In this Figure the upper traces show the EOG whilst below is the summated neural discharge. The fractional saturation of amyl acetate as stimulus is indicated. From Tucker, (1963).

Shibuya, (1969), recorded simultaneously the EOG, spike activity in the epithelium and nerve discharge in response to various concentrations of amyl acetate. Close examination of Figure (14) reveals that the onset of the EOG precedes the other activities. This is a prerequisite if the EOG is to represent a generator potential which is causative of impulse activity.

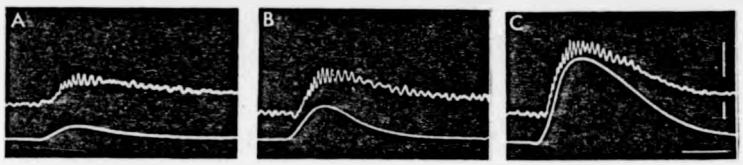
FIGURE (14)



Simultaneous recording of EOG, single cell spike activity and olfactory nerve discharge. From Shibuya, (1969).

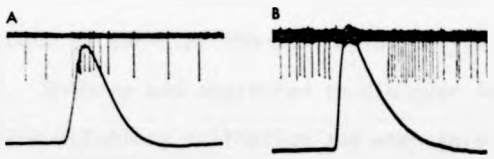
iii) The relationship between the EOG and the slow potential from the olfactory bulb is shown in Figure (15), and the EOG is compared with the activity from bulbar units in Figure (16). In the bulb, both excitation and inhibition of impulse activity is observed.

FIGURE (15)



Comparison of the EOG (lower trace) with the slow bulbar potential for responses to butanol in increasing concentration (a) through (c).
Vertical bars indicate 1 millivolt and the horizontal bar is a 1 second time mark. From Ottoson, (1959).

FIGURE (16)



From Døving, (1966), showing the effects of olfactory stimulation on bulbar units and the EOG. Unit (A) shows excitatory activity whilst the firing of unit (B) is inhibited.

iv) Finally, Plattig and Kobal, (1978), have shown that the EOG recorded from humans is matched by the psychophysical response.

These, then, are the arguments for the EOG being the generator potential of the olfactory end organ. Experimental evidence contrary to this thesis has come from Shibuya, Mozell and Takagi and co-workers.

Shibuya, (1964), found the portion of epithelium innervated by a small nerve twig dissected out from the olfactory nerve and simultaneously recorded the EOG and the afferent discharge in the nerve twig. After a piece of absorbent paper had been placed for a few minutes on the part of the epithelium innervated by the nerve twig and then removed, the EOG could no longer be obtained, but the afferent discharge still appeared. Thus the EOG had been dissociated from activity in the olfactory nerve.

Ottoson, (1971), strongly criticized the above experiment on the grounds that the remaining afferent activity might have originated from neurons in parts of the epithelium left untouched by Shibuya's treatment. Shibuya had neglected to discover the effect of treating the entire olfactory epithelium and when this was done, by Ottoson and Shepherd, (1967), both the EOG and the afferent discharge disappeared. Also, Takagi, (1967), put forth the possibility that Shibuya's treatment of the epithelium might have succeded in removing water from the mucus with a consequent change in mucus resistance that caused recording of the EOG to be unsuccessful.

Experiments by Mozell, (1962), have been quoted as evidence against the generator hypothesis because they showed that the EOG

was not always matched by the summated afferent discharge. However, Mozell himself "...emphasised that the differences occured only under very specific conditions that had to be isolated from the many conditions under which the two paralleled each other". He also pointed out that the summated discharge might be suspect "since it depends not simply on the spike frequency but also on the spike amplitude" and that "the neural responses might not have been elicited by the same mucosal regions which yielded the EOG".

Finally, experiments by Takagi and co-workers (reviewed, together with the above experiments, by Takagi, 1967 and 1969) contradicted the generator hypothesis by showing that there were circumstances under which the EOG was not correlated with the afferent discharge and bulbar activity and that the EOG did not always increase with raised odorant concentration.

However, in all these experiments ether and chloroform at high concentrations were used as odorants. These are, of course, anaesthetics and Ottoson had already shown that exposure of the epithelium to them caused diminution or abolishment of the EOG, (Ottoson, 1956).

The EOG's elicited in these experiments included positive, off, and after potentials, the origins of which will be discussed below.

In his last review, in 1969, Takagi concluded that (negative-on) EOG's "are true generator potentials".

1.2 ii) Positive EOG's and other complex potentials

Figure (17) shows the variety of potentials that, given the right conditions, may be elicited from the olfactory epithelium.

The first is the 'negative-on' EOG which has already been discussed. The second potential drawn is a monophasic positive EOG elicited, in this case, from frog by a puff of methanol. This 'positive-on' EOG was first studied by Takagi et al, (1960), although Ottoson, (1956), had sometimes observed an initial positive deflection preceding the negative EOG. He had believed this to be an artefact due to positively-charged water ions.

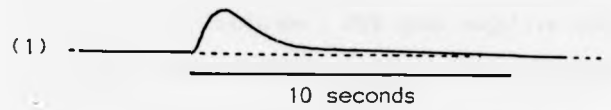
Third is an EOG from a fish (Channa argus), and is of a type discovered by Shibuya, (1960). It is a positive-going transient that appears at the cessation of stimulation (a 'positive-off' potential). Other examples of this type may be found in Gesteland et al, (1965).

The next is also an off potential, but is of electronegative polarity. This 'negative-off' EOG was first described by Takagi and Shibuya, (1959), working on frog. The example here also shows a small positive-on wave.

The final example in Figure (17) is a 'positive after potential' obtained from rabbit. This was first found by Macleod, (1959).

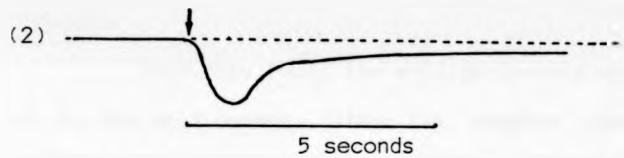
The complexity of the responses can be rationalised as a summing of the negative generator potential with, possibly, two positive processes, all three responses having different time courses.

FIGURE (17)



10 seconds

Negative-on EOG — Animal: frog; Odorant: pyridine; From Gesteland *et al*, (1963)



5 seconds

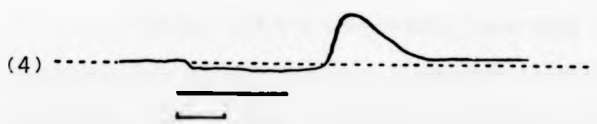
From: Gesteland *et al*, (1965)

Positive-on EOG — Animal: frog; Odorant: methanol

5 seconds

Animal: *Channa argus*; Odorant: pupa fluid; From: Shibuya, (1960)

Positive-off EOG



2 seconds

Animal: frog; Odorant: diethyl ether; From: Takagi *et al*, (1960)

Negative-off EOG

30 seconds

Animal: rabbit; Odorant: benzene; From: MacLeod,(1959)

Positive-after Potential

The potentials that can be elicited from the olfactory epithelium. Vertical scale: millivolts, negative upwards; horizontal scale: time in seconds as indicated. Stimulus indicated by thick bar or arrow.

1.2 iii) Frequency of occurence of the various types of EOG

In a survey of 122 odorants, Takagi et al. (1969,b), found that 87% elicited purely negative EOG's and 5% purely positive EOG's. Of the remainder, 2½% gave negative EOG's at low concentration that turned into positive EOG's at high concentration, and most of those left gave negative EOG's with positive after-potentials at low concentration, and, at high concentration, gave larger after-potentials with the negative EOG's increased, decreased, or remaining the same.

Thus they found the straightforward negative-on EOG to be by far the most common. Since the 'complex' responses were obtained from organic solvents, (such as chloroform, dichloromethane, and dichlorethane), or from reactive compounds, (such as formic acid and methylamine), and, since a selection of odorants from nature, rather than from the laboratory shelf, would contain fewer of these and also be at lower concentration, it must be concluded that all but the negative-on EOG are exceptional and unphysiological.

Takagi and his co-workers have made a detailed study of the various types of EOG and have succeeded in ascertaining their mechanisms and the cell-types responsible for their production.

1.2 iv) Cellular origins of slow potentials in the olfactory epithelium

Earlier on it was described how sectioning of the olfactory nerve causes degeneration of the neuronal cell population in the olfactory epithelium. Takagi and Yajima, (1964, 1965), Takagi and Wyse,(1965), and Takagi et al, (1969,b), sectioned the olfactory nerves of frogs and then followed the electrical responses as the cell structure of the olfactory epithelium changed.

They found that the negative EOG disappeared along with the neurons whilst the positive EOG survived and was often more pronounced. With complex responses the negative parts disappeared, but not the positive parts. Figure (18) shows the fate of the negative and positive EOG's, following unllateral nerve section in Rana catesbiana.

FIGURE (18), (a) and (b)

(a)

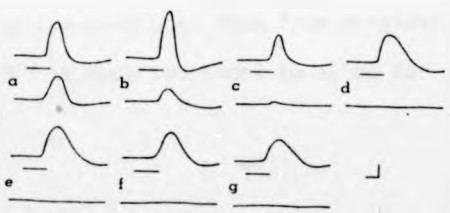
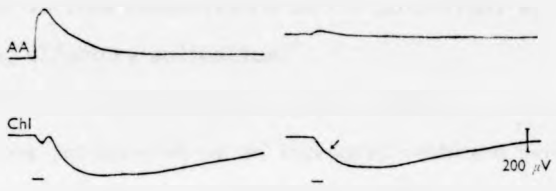


FIG. 2. Changes in amplitude of the EOG's. In each pair of records, the slow potential at the top was obtained as a control in the olfactory epithelium of the normal (left) side and the potential at the bottom was recorded in the sectioned (right) side of the same bullfrog. The responses were obtained 5 days after the nerve section in *a*, 6 days after in *b*, 7 days after in *c*, and 8 days after in *d*. Here the EOG completely disappeared. In *e*, *f*, and *g*, which were obtained 9, 10, and 12 days after respectively, no response could be found in the sectioned side (at the bottom).

(b)



Text-fig. 1. Negative and positive EOGs. The negative and positive EOGs elicited in the normal olfactory epithelium are shown on the left and the ones elicited in the degenerated epithelium are shown on the right. It is clear that the negative EOGs are not elicited at all or very small artifactual potentials are produced in the degenerated epithelium. The negative off-EOG elicited by chloroform (Chl) vapour also disappears in the degenerated epithelium and only a vestige is seen as indicated by an arrow (bottom records). AA, amyl acetate. The horizontal bars at the bottom indicate 4 sec.

Disappearance of the (negative-on) EOG following olfactory nerve sectioning in frog.

(a) From Takagi and Yajima, (1965)
(b) From Okano and Takagi, (1974)

The conclusion to be drawn is that the negative EOG is generated by the olfactory neurons whilst the positive potentials are due to the supporting cells. The position of the basal cells deep in the epithelium precludes them from consideration because the latency of the EOG* is much too short to allow for diffusion of odorants to them.

* 200 milliseconds, Ottoson, (1956).

1.2 v) Ionic mechanisms

Takagi et al, (1966, 1968, 1969,a,b) investigated the ionic basis for EOG generation by studying the effects of Ringer's solutions of various compositions on the potentials elicited from excised frog olfactory epithelium.

They concluded that the negative EOG is generated by influx of sodium ions accompanied by an increased membrane permeability to potassium ions, whilst the ionic mechanism of positive potentials is mainly dependent on movement of chloride ions, again with a contribution from potassium ions. They found that the ionic basis of positive potentials was the same in both normal and degenerating epithelium and they compared the positive EOG with inhibitory potentials of other receptors.

Okano and Takagi, (1974)* used electron microscopy to examine the effects of odour stimulation at a cellular level and found that the positive EOG to chloroform was accompanied by vigorous secretory activity in the supporting cells**. The same effect was not found when the epithelium was exposed to amyl acetate or other compounds which elicit negative EOG's. They ascribed the slow positive potential to this secretory activity, (i.e. the positive after-potential which would only be discernable after the negative EOG is switched off).

So we now have a picture of the negative generator potential with a hyperpolarizing positive potential and a slow positive secretory potential combining to create complex EOG wave-forms.

* Quoted as unpublished results in Takagi, 1969 and 1971

** Note: In the animal used, frog, the supporting cells are packed with secretory droplets, a feature not found in the mammalia.

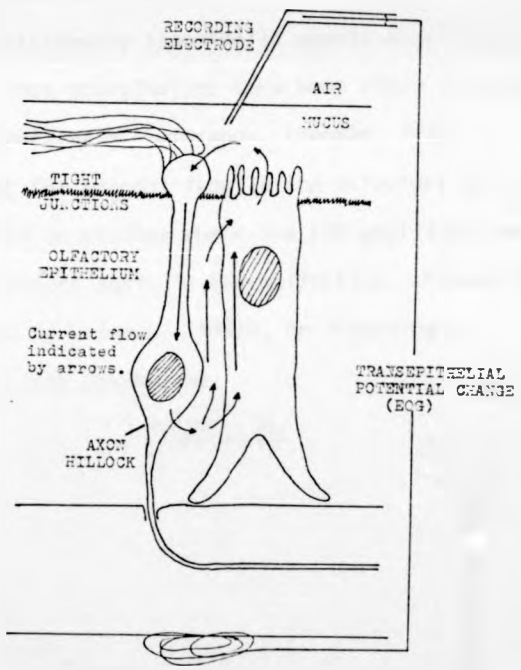
1.2 vi) Generator currents in the olfactory epithelium

The mechanism by which nerve impulses are generated in the olfactory epithelium is as follows.

Odorous stimulation results in the opening of ion-gates on the sensory region of the neuronal membrane. (Hence the changes in impedence monitored by Gesteland et al, (1965), Figure (9)). Na^+ ions are the ionic species furthest from equilibrium across the cell membrane and they flow down an electrochemical gradient into the neurons. This influx of positive charge means that the electrode in the mucus which is recording EOG's observes a negative-going potential change.

The flow of sodium ions, the generator current, passes down the neurons discharging the membrane potential. At a certain point on the neuron, believed to be the axon-hillock, the cell membrane begins to respond to the depolarization by generating an action potential which travels down the axon. In the wake of the action potential the resting potential of the membrane is restored by efflux of K^+ ions, so before another impulse can be initiated a further flow of generator current is required. In this way, the magnitude of the generator current can control the frequency of discharge of the neuron. The circuit is completed by current flow from the axon-hillock back to the mucus layer which must be via the supporting cells, since tight-junctioned complexes seal off the mucus layer from extra-cellular spaces within the epithelium.

FIGURE (19)



Based on Gesteland, (1971), showing the flow of generator current through the epithelium.

The EOG will have a dependency on the transepithelial resistance,

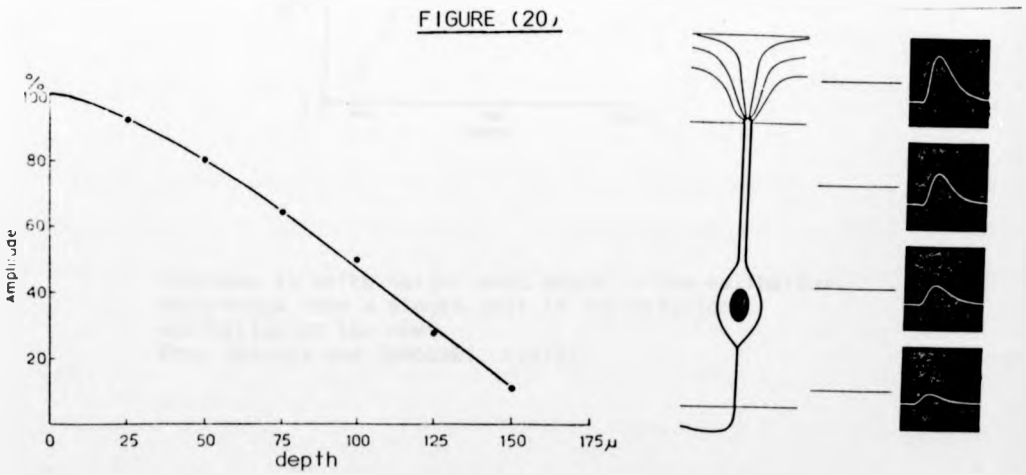
$$(V_{EOG} = I_{\text{generator current}} \times R_{\text{transepithelial resistance}})$$

see Thurm, (1972). The transepithelial resistance will be mainly dependent on that of the cell membrane of the supporting cells, because this will have a higher resistance than that of the external milieu. So the physical status of the supporting cell membrane will influence the magnitude of the EOG.

The EOG elicited by anaesthetic agents should be viewed in the light of this since anaesthetics have been shown to cause changes in the physical properties of membranes, (Seeman, 1972).

This model for current flow in the olfactory epithelium has been based partly on studies where the EOG amplitude has been measured as a function of depth in the epithelium. Figure (20) shows the results obtained by Ottoson, (1956), on inserting a microelectrode into frog epithelium.

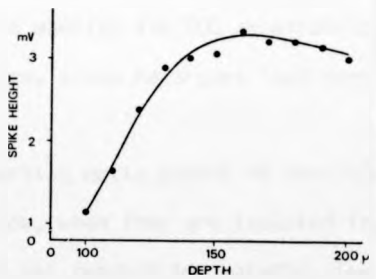
FIGURE (20)



Variation in EOG peak height with depth in the epithelium. From Ottoson, (1956).

Shibuya and Tonosaki, (1972), have determined the depth at which impulses were initiated in newt epithelium. Figure (21) shows their results where a maximum spike height was obtained at the depth of the neuronal cell bodies, 150 microns below the surface. This roughly coincides with the depth at which the EOG began to disappear in frog epithelium in Ottoson's experiment above and this ties in with the model.

FIGURE (21)



Increase in spike height with depth in the epithelium.
Recordings from a single unit in the olfactory epithelium of the newt.
From Shibuya and Tonosaki, (1972).

1.2 vii) The site of transduction

The cilia on the olfactory neurons are the morphological elements most likely to be responsible for transducing olfactory stimulation into nervous activity.

Ciliation increases the membrane area of the neurons many-fold and the cilia are the first features in the epithelium that an odorant molecule would encounter, since their position in the mucus places them closest to the external environment. Figure (20) shows that the EOG has maximum amplitude at the surface of the mucus where the cilia lie. They are also morphologically distinct from motile cilia which may indicate specialization and Menco et al, (1976), and Menco, (1977, 1978), have shown that they bear membrane particles at high density which are putative receptor sites. Ottoson, (1971), has used Shibuya's (1964), experiment of applying absorbent paper to the epithelium to abolish the EOG as evidence that the cilia are the sensory receptors, since he argues that such treatment must remove cilia.

The supporting cells cannot be the receptor sites in the olfactory organ since, when they are isolated in the degenerating epithelium, they do not respond to odorants, (as opposed to anaesthetic compounds). If the EOG represented the receptor potentials of the supporting cells, when measuring the EOG responses as a function of depth, a change of sign would be observed as the apical surface of the supporting cell was penetrated. This has not been found in studies by Ottoson, (1956), Byzov and Flerova, (1964), Tucker and Shibuya, (1965). (Supporting cells account for the major portion of the surface area of the cell layer in the epithelium. An

electrode descending through the mucus would most likely penetrate the epithelial surface through the apical membrane of a supporting cell).

The terminal swellings of the neurons are not excluded from a role in transduction and may perform the function of detecting odours with the cilia.

However, it has not yet proved possible to isolate the components of the olfactory epithelium and thus unequivocally to allot functions to these. Therefore the position of the cilia as the site of olfactory transduction remains a matter for debate.

1.3 Processing of Olfactory Information

1.3 i) Intensity coding

Neural coding for the intensity of an olfactory stimulus follows the same general pattern found in other sensory modalities: the frequency of impulse generation in the olfactory neurons increases with odorant concentration. The concentration ranges covered by individual neurons was found to vary from 0.5 to 2.0 logarithmic steps by Mathews, (1972), whereas the sensitivity range of the epithelium as a whole, as monitored by the EOG, covers 4.0 to 5.0 logarithmic steps, (from data in Poynder, 1974,a,b). This is because the neurons vary in sensitivity and, as the stimulus concentration is increased, units with lower sensitivities are recruited to contribute to the overall response.

The extreme sensitivity for which the olfactory system is renowned is made possible by a high degree of neural convergence. The convergence ratio of olfactory neurons to secondary cells in the olfactory bulb has been shown to be about 1,000 : 1 in rabbit, (Allison and Warwick, 1949), in bats, (Bhatanger and Kallen, 1975), and in burbot, (Gemne and Døving, 1969). The significance of this has recently been discussed by van Drongelen, (1978), van Drongelen et al, (1978), and Holley and Døving, (1978). A small increase in the firing rate of the peripheral neurons, undetectable at the single cell level, becomes a significant event at the level of the bulb where all the responses are integrated. Such a mechanism functions best if the sensory neurons have a low level of resting activity, (van Drongelen et al, 1978), and this was found in single unit studies. Figures for the average rate of spontaneous discharge of frog olfactory neurons

include 3.5 ± 2.5 per minute , (O'Connell and Mozell, 1969), 6 per minute, (Daval et al, 1972) and 3 - 7 per minute, (Altner, 1974). Most authors report 'silent' units, which only fire in response to stimulation, and also a smaller number of units with a higher spontaneous activity of, for example, 134 ± 25 spikes per minute, (O'Connell and Mozell, 1969). These may respond by having their activity suppressed. For comparison, a stimulated cell will have a firing rate of up to 1,200 spikes per minute, (Revial et al, 1978,a).

Using convergence to obtain sensitivity demands a large number of receptors, but does not require individual receptors to have high sensitivity. Insects, having olfactory organs of limited size, use specialist, low-threshold receptors for detecting, for example pheromones. Such receptors tend to exhibit high specificity. That vertebrates do not need such specialists for sensitivity is helpful in understanding the way quality is discriminated by them.

1.3 ii) Quality coding

Two mechanisms might be used for differentiating between odours: spatio-temporal patterning using the organ as a whole, or selectiveness at a single cell level. They are not mutually exclusive and experimental evidence supports both.

Spatio-temporal patterning is observed in the electrical activity of the olfactory bulb, (e.g. Adrian, 1953, Moulton, 1965, and Freeman, 1978). Adrian suggested that the patterning was projected from the peripheral level.

A spatially organised projection of nerve fibres from epithelium to bulb has been demonstrated for the medio-lateral plane in frog,(Costanzo and Mozell, 1976) and hamster (Costanzo and O'Connell, 1978), but projection in the antero-posterior plane appears to be weak, (Moulton, 1976).

Spatio-temporal patterning in the summated discharge of olfactory nerve fibres has been demonstrated by Mozell, (1964,a,b, 1966, 1967, 1969, 1970, and reviewed in 1971). The medial : lateral ratio of activity in nerve branches was shown to be odorant dependent, suggesting that odorants distributed themselves differentially across the mucosa. This was proved by following tritiated odorants, (Hornung and Mozell, 1977). An analogy was drawn between the spatio-temporal analysis of odorants by the olfactory epithelium and chromatography. Mozell and Jagodowicz, (1973), showed that a frog's olfactory sac could be used to separate odorants instead of a chromatography column.

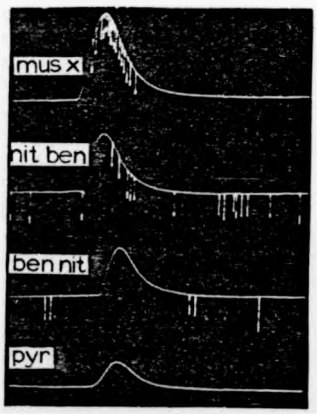
Thus spatio-temporal patterning depending on the physiochemical properties of odorants has been shown to be generated

in the epithelium and projected to the bulb.

There also exists intrinsic spatial patterning since the ECG recorded from different areas of the epithelium shows differential responses to odorants,(Daval and Leveteau, 1969, Mustaparta, 1971, Daval et al, 1972 and Kauer and Moulton, 1974). This suggests a non-homogeneous distribution of receptors across the epithelium with respect to their specificities.

Gesteland et al, (1963), showed that single units responded differentially to odorants. For example, the unit shown in Figure (22) exhibits a greater discharge rate in response to a musk than to other odorants tried.

FIGURE (22)



Oscilloscope traces of unit activity superimposed on the EOG. The unit responds to musk xylene and slightly to nitrobenzene. The other odorants are benzonitrite and pyridine.
From Gesteland et al, (1963).

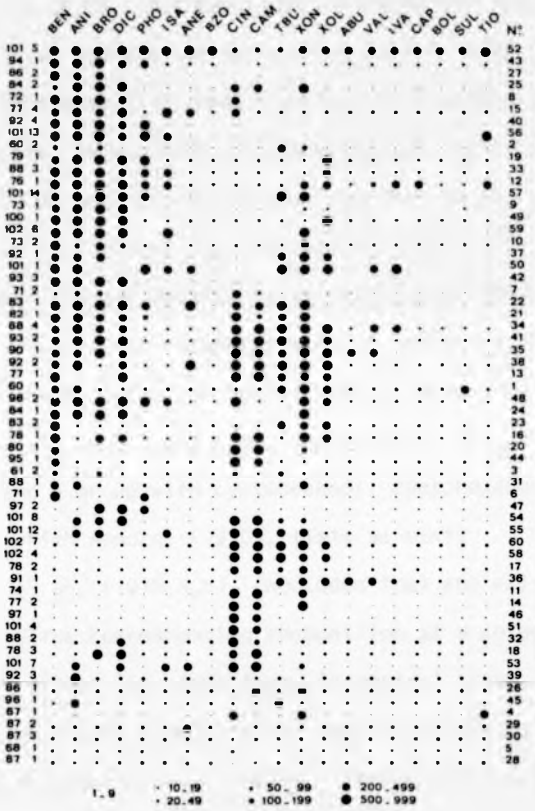
Another unit might respond to one of the other odorants and not to the musk. Nevertheless, the specificities were always found to be overlapping. The model that Gesteland et al, (1963), proposed to explain their results was that the neurons have receptor sites of different specificities distributed amongst themselves in varying combinations.

Recording the activity of units to a battery of test odorants has been used to show how the specificity of the receptors varies. Figure (23) from Revial et al, (1978,a) shows the results from such a study.

Key to Figure (23) overleaf

Abbreviation	Odorant
BEN	Benzene
ANI	Anisole
BRO	Bromobenzene
DIC	1,3-Dichlorobenzene
ISA	Anisaldehyde
ANE	Anethole
BZO	Benzophenone
CAM	dl-Camphor
CIN	Cineole
XOL	Cyclohexanol
XON	Cyclohexanone
TBU	Tert-butyl alcohol
ABU	n-Butyric acid
VAL	n-Valeric acid
IVA	iso-Valeric acid
CAP	Caproic acid
TIO	Thiophene
PHO	Thiophenol
BOL	Butanethiol-1
SUL	Diethylsulfide

FIGURE (23)



Diagrammatic representation of unit responses to a battery of odorants; units are identified by the number of the frog followed by the number of the unit. The odorants used are shown at the tops of the columns, and a key to their identification is given above. Dot sizes represent spike frequencies (spikes per minute).
From Revial et al, (1978,a).

The degree of receptor specificity is found to vary widely, with receptors responding to from 0 to 70% of test odorants, (Holley, 1974). The average response rate was found by Duchamp et al, (1974) to be 18.5%. Mathews, (1972), found that specificity was enhanced at lower odorant concentrations by 29 - 100%. Although intensity coding operates with quality coding at a single cell level, absolute specificity, independent of concentration, is exhibited.

Various odorants can be grouped together by the responses that they elicit from single cells. Examination of Figure (23) shows that cineole and camphor are not usually differentiated by the receptors. Statistical analysis of unit responses allowed Revial et al, (1978,b), to distinguish three groups of odorants: camphoraceous, with camphor and cineole; aromatic, which was comprised of odorants containing a benzene ring; and a group with cyclohexanol, cyclohexanone and t-butyl alcohol, which Amoore, (1970), lists as minty.

Revial et al, (1978,a,b), concludes that the existence of odorant groups implies corresponding recognition at a molecular level by receptor sites or, as they term them, 'acceptor' sites. Holley, (1974), believes that such receptors are membrane proteins with low affinity and low specificity binding. The multiple sensitivities of the neurons can be explained by the distribution of the receptor proteins and consequently the neurons themselves cannot be simply categorised. Evidence that the receptors are proteins has come from group-specific and affinity labelling experiments, (Getchell and Gesteland, 1972, Menevse et al, 1976, 1977,a). Low specificity of binding exhibited by these receptor proteins would explain how the nose has the ability to react with virtually every volatile compound.

Low specificity would imply a low affinity of binding and thus low sensitivity. It has already been described, though, how such a lack of sensitivity at the peripheral level can be compensated for by processing. A low affinity of binding would also help in the removal of odorants.

The olfactory system in vertebrates thus seems to have evolved in a way such that the needs for discrimination and sensitivity are reconciled to achieve a high degree of both.

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 Choice of Experimental Animals

For *in vivo* experiments frogs were used since they have been the animal of choice for electrophysiological studies in olfaction. The results presented in this thesis may therefore be compared directly to most previous work. Frogs (*Rana temporaria*) have been shown to respond behaviourally to odorants, (Müller and Kiepenhever, 1976), as have other amphibians: *Xenopus*, (Kramer, 1933), and *Triton*, (Matthes, 1927).

For the experiments in Chapter 5, where results from the frog are compared to those from a mammal, sheep tissue *in vitro* was used. No difficulty was encountered in recording EOG's from rat and calf tissue, but availability, cost, and ease of dissection led to sheep being chosen.

2.2 Animal and Tissue Preparation

i) Animal preparation for *in vivo* recordings

For *in vivo* recordings the common frog, *Rana temporaria*, was used. The animals, (male and female, *ca.* 20g) were obtained from Gerrard Biological Supply, Sussex, and on delivery were placed in a cold room maintained at an average temperature of $+3^{o}C$. They were kept at room temperature for at least two hours, and usually overnight, prior to an experiment. The frogs were anaesthetised by partial immersion in a 10% w/v aqueous urethane solution until reflex activity was lost, and then immediately washed with tap water to prevent further urethane absorption.

The frogs were rigidly clamped in a perspex holder with a mouth bar and ear screws, and body-parts were covered with a moistened tissue. Under a dissecting microscope the left olfactory cavity was opened by removal of the skin, cartilage and dorsal olfactory epithelium. The blood circulation was checked, since a good flow seems necessary to enable stable EOG recording over a period of hours. The holder was then locked into position beneath the odour applicator.

2.2 ii) Tissue preparation for in vitro recordings

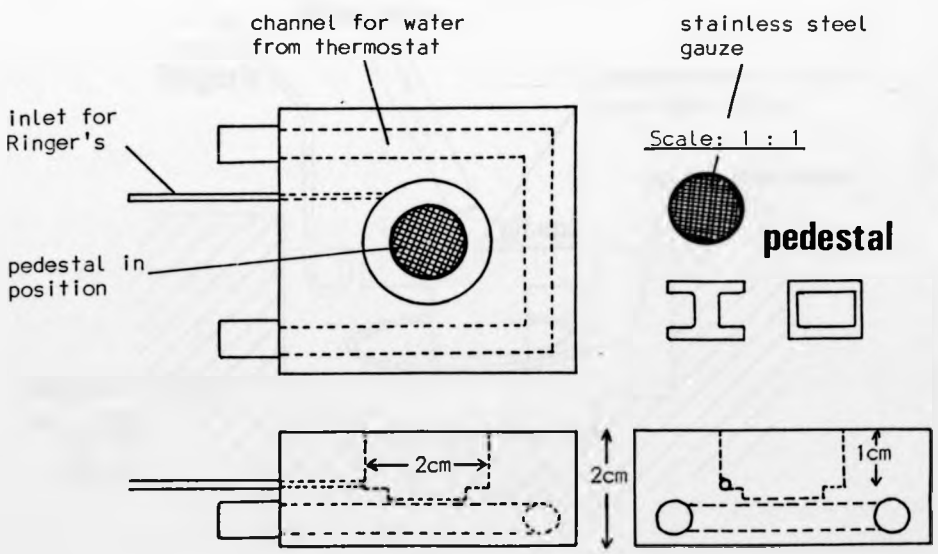
Sheep's heads were obtained at a local abattoir immediately following death. They were sagitally bisected with a band saw, packed in ice and brought back to the laboratory. The nasal septum was removed to expose the ethmoturbinates, as shown in Figure (1). A piece of olfactory epithelium, 4 - 5 mm square was carefully dissected from one of the ethmoturbinates and placed in the tissue cell as shown in Figure (25). Typically the whole operation could be completed within 15 - 20 minutes of death.

The procedure for calf was the same. Wistar rats and frogs were killed and the excised olfactory epithelium, together with the underlying bony tissue, (i.e. a whole turbinate or olfactory eminence), was placed in the tissue cell within 2 - 3 minutes of death.

2.3 Maintenance of Olfactory Epithelium In Vitro

The excised tissue was continuously perfused with Ringer's solution. For mammalian tissue Ringer Locke, pH 7.2 - 7.4 was used and for frog tissue Frog's Ringer, pH 7.0 - 7.2, (from Data for Biochemical Research, ed. Denton et al, Oxford University Press). A constant stream of deodorised, humidified air was played over the surface of the epithelium and this arrangement permitted the tissue to be kept in active condition throughout the course of a day. The plans for the tissue cell, which was purpose-built for in vitro EOG recordings, are shown in Figure (24). It was made from high quality stainless steel and channels drilled through the block for thermostatted water to circulate in allowed the temperature of the preparation to be controlled.

FIGURE (24)

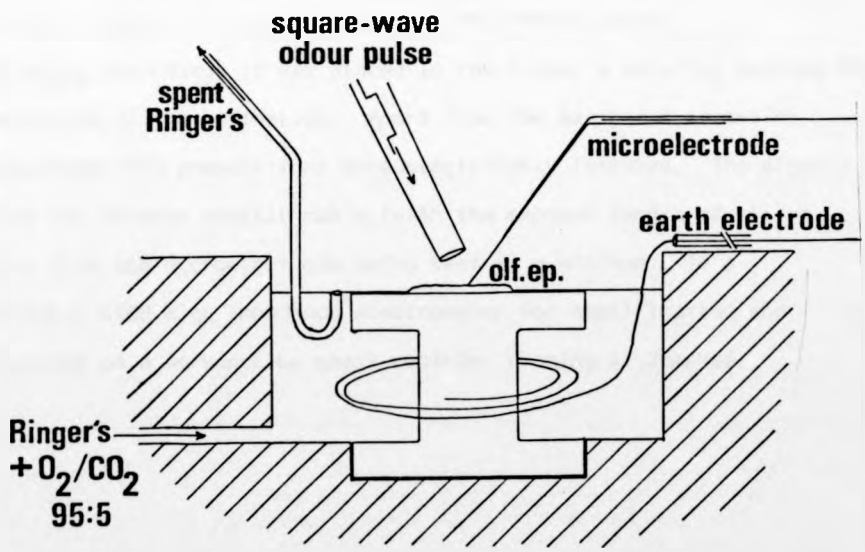


Tissue cell of own design for maintaining olfactory epithelium in vitro. Made entirely from stainless steel.

The inlet and the removal tube for the Ringer's solution were disposed to give active mixing in the tissue cell. The Ringer's solution was aerated in a 100cm glass column with 95% O_2/5% CO_2 forced through a sintered glass inlet at the bottom. A reservoir feeding into the top of the column gave a constant head and the Ringer's solution was led off from the bottom of the column through PTFE tubing to the tissue cell at 1-2mls/min. To prevent microbial growth the equipment was regularly cleaned and Ringer's solutions were only kept for a short while and stored concentrated at +4°C.

Figure (25) shows the tissue cell in operation. the level of Ringer's solution was regulated by raising or lowering the removal tube and was adjusted so that the epithelium was half submersed.

FIGURE (25)



Tissue cell in operation.

2.4 EOG Recording

The microelectrodes were pulled from 1mm i.d. pyrex tubing to give a tip diameter of 5 - 25 microns and were filled with Ringer's solution plus 1.5% w/v agar. Connection was made through a chlorided silver wire. The electrodes had an impedence of *ca.* 2 megohms and lasted for several months. When stored they were kept at +4°C, in the dark, with the tips in Ringer's solution. For recording, the tip of the electrode was placed just touching the surface of the mucus with the nozzle of the applicator 2 - 3mm away and directed straight at the point of contact. In frogs the microelectrode was positioned at the peak of the olfactory eminence. A micromanipulator was used to manoeuvre the microelectrode which, once positioned, was held rigidly in place. The earth electrode was also a chlorided silver wire. For *in vivo* recordings it was wrapped in Ringer's-soaked muslin and placed in the frog's mouth. For *in vitro* recordings it was placed in the Ringer's solution bathing the underside of the epithelium. Apart from the earth and recording electrodes the preparations were electrically isolated. The signals were led through coaxial cable,(with the exposed length of silver wire from the microelectrode being kept to a minimum), to a Keithley 610B high impedence electrometer for amplification and recorded on a Servoscribe chart recorder running at 2mm/sec.

2.5 Stimulus Control

For the experimental work a defined, reproducible stimulus was essential.

2.5 i) Olfactometry

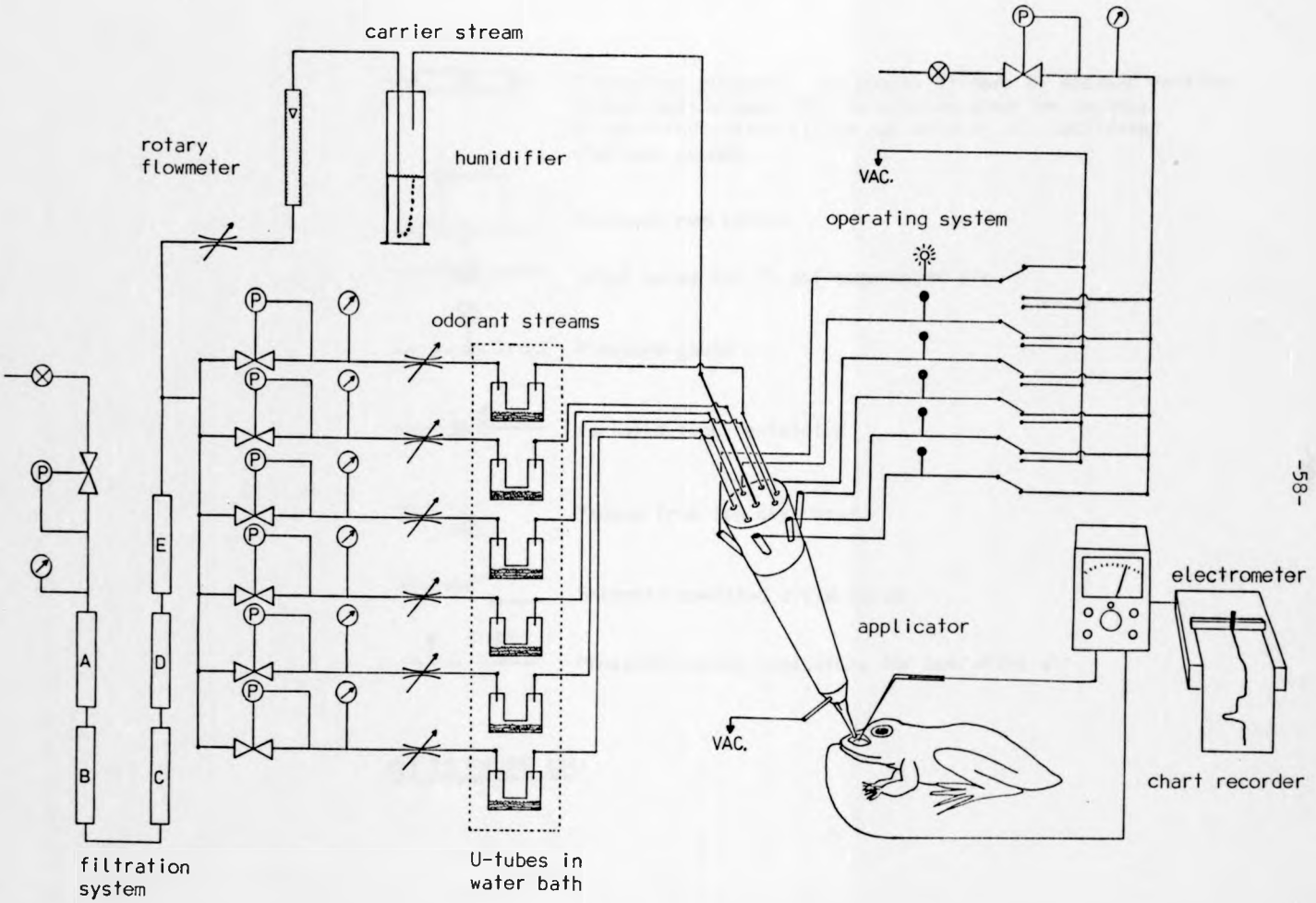
A six-channel flow-dilution olfactometer similar to that described by Poynder, (1974,a), was built. Its layout and the general experimental set-up are shown diagrammatically in Figure (26). The components used were mainly obtained from Festo Pneumatics Ltd., Catherine Wheel Road, Brentford, Middlesex, and these permitted the olfactometer to be made into a compact form, (30cm high x 67cm long x 16cm deep).

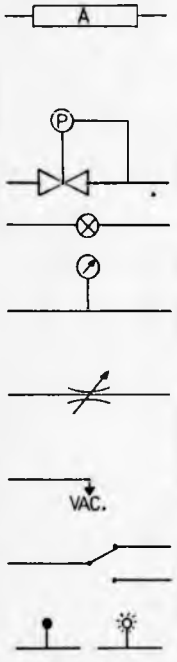
Compressed air was filtered, dried, and deodorised and split into two streams. One stream was adjusted to give a flow rate of 100mls/min and rehumidified: this was the carrier stream. The other stream of dry, clean air was split in to six channels with independently adjustable pressures, and flow rates, (0-25p.s.i. and 0-5 mls/min respectively). These streams were passed over pools of liquid odorant held in specially made U-tubes. The surface of odorant presented to the air streams was 1 x 8 cm. The U-tubes were held in a water bath whose thermal inertia served to keep the vapour pressures of the odorants steady. U-tubes were cleaned in chromic acid, washed in double-distilled water and ethanol, and baked until odourless. The P.T.F.E. tubing connecting the U-tubes was replaced when the odorant to be carried was changed and the stainless steel tubing inside the applicator was flushed out with clean air.

FIGURE (26)

Diagrammatic representation of stimulus delivery system.

Key on page 59.


carrier stream
rotary
flowmeter
humidifier
VAC.
operating system
odorant streams
E
A
D
B
C
applicator
electrometer
VAC.
chart recorder
filtration
system
U-tubes in
water bath



Filtration elements A: course filter; B: Whatman in-line filter unit (Gamma 12); C: calcium chloride column; D: self-indicating silica gel column; E: activated charcoal column

Pressure regulation

Inlet valve for 75 psi compressed air

Pressure gauge

Variable flow restrictor

Vacuum from tap aspirator

Solenoid operated 2-way valve

Pressure/vacuum indicators for operating air

KEY TO FIGURE (26)

2.5 ii) Stimulus switching

The applicator part of the apparatus was identical to that described by Poynder, (1974,a) and Bostock, (1974,a), Figure (27). Its valve system was pneumatically operated and allowed one or more streams of odorised air to be rapidly switched into the carrier stream to give a square-wave stimulus profile. The flow of odorised air was kept to less than 10% of that of the carrier stream, which went forward onto the preparation at 50mls/min. Mechanical artefacts were thus avoided.

A second applicator of novel design was also made, but it proved less reliable than the one designed by Poynder, which was therefore used for the experiments in this thesis. However, the second applicator had some superior features and so is described in Appendix I.

FIGURE (27)

(a) Detail of applicator showing switching system .
Channel 1 off, odorant removed by suction.
Channel 4 on, odorant carried forward by humidified air stream.

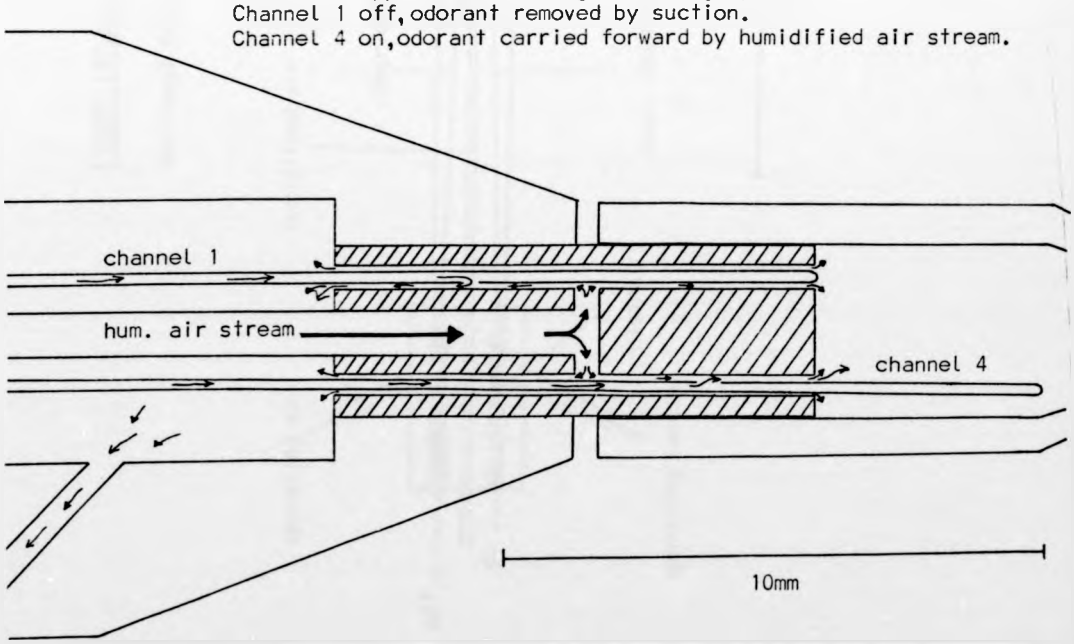
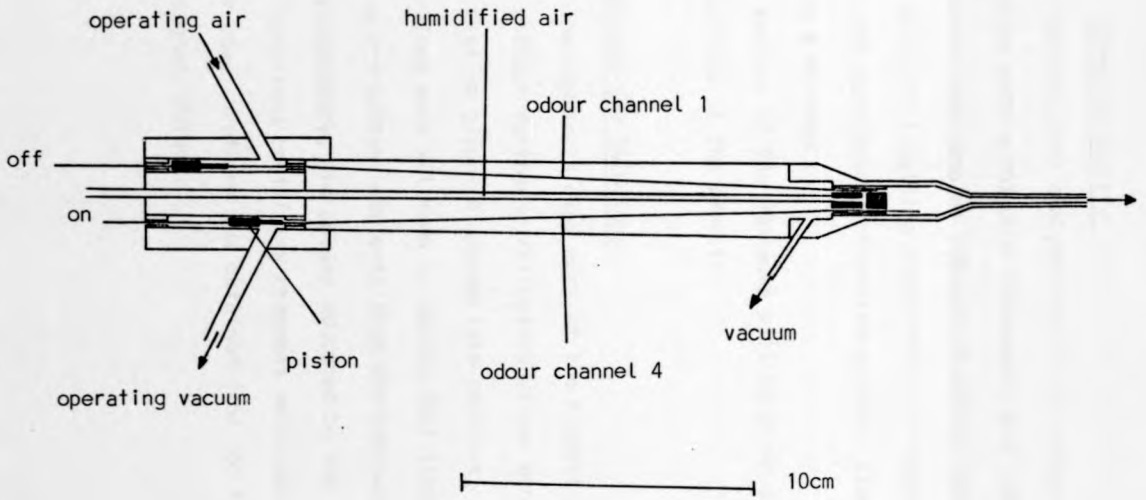


FIGURE (27,b)

Sectional drawing of six-channel odour applicator



2.5 iii) Stimulus monitoring

The characteristics of the stimulus, (wave-form and concentration), were monitored with a gas sensor under development in our laboratory. Concentration measurements of vapour phase odorants will be expressed in moles of odorant/litre of air, as proposed by Laffort, (1963).

2.5 iv) Stimulus quality

The odorants were analysed by G.L.C. using a Perkin-Elmer F-11 Chromatograph with a 20% DEGS Chromasorb W-HP 100 - 120 mesh column, N_2 carrier, and samples 10% v/v in diethyl ether.

For affinity labelling experiments in Chapter 4, the odorants used were pure unless otherwise stated. Elsewhere, 98% was accepted as a minimum.

The sources of the odorants will be given in the appropriate sections of the results.

2.6 Chemicals and Solutions

All the chemicals used were of the highest purity available and were used without further purification unless otherwise stated. Their sources will be given in appropriate sections.

Solutions were all made in double-distilled water using inorganic salts and buffer nutrients from BDH Chemicals, Poole, Dorset. Where necessary, the pH was adjusted by the addition of HCl or NaOH. Solutions containing reagents were made up immediately prior to their use. Urethane was obtained from the Aldrich Chemical Company, Gillingham, Dorset.

CHAPTER 3 A STRUCTURE ACTIVITY RELATIONSHIP STUDY OF SIMPLE ALKYL ESTERS

3.1 Introduction

The odour description 'fruity' is included in several odour classification systems such as those of Zwaardemaker, (1895), Henning, (1915), and Harper *et al*, (1968). Most fruity-smelling compounds seem to be esters, although esters are not necessarily fruity. In electrophysiological experiments in olfaction, simple alkyl esters, for example pentyl acetate* and ethyl butyrate, which do have fruity odours, are very frequently used as odorous stimulii because of their efficacy in producing responses from the olfactory epithelium and bulb. However, in studies on structure-activity relationships in olfaction, such compounds are scarcely mentioned.

Since the affinity labelling experiments which will be described in the next chapter were primarily concerned with esters, it was deemed necessary to investigate structure-activity relationships for these compounds.

* usually referred to as amyl acetate.

3.2 Materials and Methods

Ethyl butyrate, isobutyric acid, pulegone, trimethyl acetic anhydride, pentanoic acid and iso-pentanoic acid were obtained from the Aldrich Chemical Company Ltd., Gillingham, Dorset; ethyl acetate, methyl acetate, pentyl acetate, iso-pentyl butyrate, iso-propyl butyrate, ethyl formate, methyl formate, pentyl formate and propionic anhydride from BDH Chemicals, Poole, Dorset; butyl acetate, butanol, iso-butanol, t-butanol, iso-butyric acid and formic acid from Fisons Chemical Company, Loughborough; sec-butanol, and butyric acid from Hopkin and Williams Ltd., Chadwell Heath, Essex; pentanol and iso-pentanol from May and Baker Ltd., Dagenham, Essex.

Butanol, iso-butanol, sec-butanol, t-butanol, pentanol and iso-pentanol were redistilled before use.

Table III presents odour descriptions for 80 esters. Amoore, (1962, 1970), Arctander, (1969), the 1970 Haarmann and Reimer Compendium, and Moncrieff, (1951), provided descriptions for 51 of the compounds, samples of 10 of which were obtained commercially. A further 38 compounds were synthesised. In the Table the odour of each compound is described by a descriptor, or a list of descriptors, with the appropriate reference(s).

Esters were synthesised by refluxing the alcohols with the acids or anhydrides, (Vogel, 1956). The products, which are water insoluble, were washed with dilute aqueous $NaHCO_3$ solution to remove starting materials, dried by shaking with fused $CaCl_2$, and redistilled until at least 99% pure as judged by G.L.C. Compounds were identified by ^1H NMR (Perkin-Elmer R12) or by mass spectrometry (A.E.I. MS 1073, courtesy of Mr. M. Farmer).

Odour descriptions were made by me on three separate occasions, notes were compared, and then quality judgements were checked against the opinions of three other people, (2 male and 1 female). Pulegone was used as a reference for mintiness. It should be noted that the sec-butyl esters described are racemates.

3.3 Results

TABLE III

Odour Descriptions of Simple Alkyl Esters

Key to references

a Amoore, (1962, 1970)

b Arctander, (1969)

c Haarmann and Reimer Compendium, (1970)

d Moncrieff, (1951)

e Synthesised by D. J. Squirrell

f Synthesised by M. J. Farmer

g Synthesised by S. Speed

h Compound obtained from Fisons

i Compound obtained from Aldrich

j Compound obtained from BDH

See Appendix II for odour descriptions of additional compounds

	Methyl	Ethyl	Propyl	Butyl	Pentyl	iso-Pentyl	iso-Butyl	iso-Propyl	sec-Butyl	t-Butyl
Formate	Ethereal[abj]	Pungent[c] Fruity[bc] Ethereal[bj] Rum[b]	Ethereal[b]	Fruity[b] Ethereal[b] Rum[b]	Fruity[cj] Ethereal[j] Pungent[c] Almond[j]	Fruity[b]	Ethereal[b] Rum[b] Raspberry[b]	Ethereal[b]	Almond[g] Ethereal[g] Fruity[g]	Minty[e] Ethereal[e]
Acetate	Pungent[c] Fruity[bcj] Ethereal[abj] Fragrant[d]	Fruity[bcj] Fragrant[d]	Fruity[bcdf]	Fruity[ch]	Fruity[cdj]	Fruity[bcd]	Fruity[bcf] Ethereal[b]	Fruity[bcf] Ethereal[bf] Minty[f]	Fruity[f] Minty[f] Ethereal[f]	Minty[f]
Propionate	Fruity[bc] Ethereal[b] Rum[c]	Fruity[bcf] Ethereal[b] Rum[b]	Fruity[bcf] Rum[b]	Fruity[e]	Fruity[g]	Fruity[bc]	Fruity[bd] Ethereal[b] Rum[b]	Fruity[bf] Minty[f] Ethereal[b]	Minty[e] Fruity[e]	Minty[f]
Butyrate	Pungent[c] Fruity[bc] Ethereal[b]	Fruity[bcdi]	Fruity[bcd]	Fruity[bc]	Fruity[b]	Fruity[bcj]	Fruity[bc]	Fruity[bcjf] Slightly minty[fj] Pungent[b]	Fruity[e] Minty[e]	Minty[e] Earthy[e]
Pentanoate	Fruity[b] Ethereal[b]	Fruity[b] Ethereal[b]	Fruity[b] Ethereal[b]	Fruity[b]	Fruity[b] Ethereal[b]	Fruity[e]	Fruity[b]	Fruity[b]	Fruity[e]	Minty[e] Fruity[e] Earthy[e]
iso-Pentanoate	Fruity[b] Ethereal[b]	Fruity[c]	Fruity[b]	Fruity[bc]	Fruity[e]	Fruity[bcd]	Fruity[bc] Ethereal[b]	Fruity[b]	Fruity[e] Minty[e]	Minty[e] Earthy[e]
iso-Butyrate	Fruity[b] Ethereal[b]	Fruity[bc]	Fruity[b]	Fruity[b]	Fruity[e]	Fruity[b]	Fruity[ab]	Fruity[be] Minty[e]	Minty[e] Slightly fruity[e]	Minty[e] Earthy[e]
tri-Methyl acetate	Minty[f]	Minty[f]	Minty[e]	Fruity/ Minty[g]	Fruity[g] Slightly minty[g]	Fruity[e] Minty[e]	Minty[e] Fruity[e]	Minty[e]	Minty[g]	Minty[f]

Of the compounds whose odours are described in Table III, 80% (64) have the description 'fruity' applied to them, 71% (57) being predominently fruity in character. Qualifying terms were also used: banana, pineapple, apple, pear (drops), apricot and peach. However, between the various sources of odour descriptions these similies were used with little consistency except for 'apricot' and 'peach' which were confined to compounds with one or both alkyl groups $\geqslant C_4$ and were used to describe a floral note which modified the fruity component.

Addendum

"Iso-butyl iso-butyrate ... smells fruity to some people and minty to others."

J. E. Amoore Chem. Sens. & Flav. 2 p.267-281 (1977).

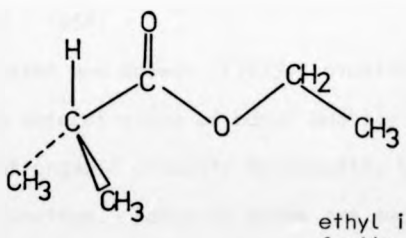
3.4 Discussion

The esters have been arranged so that non-fruity compounds lie at the edges of the Table to permit molecular features which cause a change in odour quality away from fruitiness to be identified.

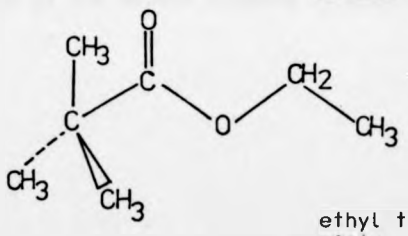
The most dramatic change is the appearance of an odour of peppermint. In the series iso-propyl, sec-butyl, t-butyl, there is a gradation in the predominence of this quality over fruitiness, whilst from the iso-butyrates to tri-methyl acetates there tends to be a shift.

Ethyl iso-butyrate is quoted by two sources as being fruity. If the α-hydrogen is substituted for a methyl group we get ethyl tri-methylacetate which smells like 'mint imperial' sweets. Figure (28) shows the structure of the two compounds.

FIGURE (28)



ethyl iso-butyrate
fruity



ethyl tri-methylacetate
minty

How such a seemingly small structural variation can cause a completely different odour quality to emerge becomes evident if space-filling models of the compounds are made, when it can be seen how the carbonyl function of ethyl tri-methylacetate is occluded by its t-butyl group whereas the carbonyl function of ethyl iso-butyrate is still exposed.

Taking the series iso-propyl, sec-butyl, t-butyl, the degree of mintiness and loss of fruitiness is correlated to the bulkiness of the side group.

If we assume that the fruity odour of an ester depends on its binding to a receptor site by the carbonyl group, then steric hindrance of such binding by a bulky side group close to the carbonyl explains the loss of the fruity odour.

Why the sterically hindered esters shown should then gain a minty odour must be due to their molecular shapes, but the scope of this study is too small to permit speculation on the determination of mintiness and the reader is referred to the series of papers by Amoore, (1952 - 1964).

Schafer and Brower, (1975), studied the role of functional groups in the determination of odour quality by investigating the ability of organic chemists to classify unknown odorants in terms of chemical function. Table IV shows the success rates for the identification of the eleven classes of chemical employed in the study.

TABLE IV Success Rates in the Identification of Odorants by Chemical Function

Chemical Class	% Correct Identification	Recognisability
Amines	87	
Esters	64	
Phenols	62	"Highly recognisable"
Sulphur compounds	61	
Carboxylic acids	53	
Ketones	42	
Aldehydes	42	"Marginally recognisable"
Hydrocarbons	36	
Alcohols	25	
Ethers	20	"Nearly or totally unrecognisable"
Halides	16	

Esters seem therefore to be one of the more easily recognisable chemical functions.

Table V gives a breakdown of the chemical classes to which the esters were ascribed. The percentages are calculated from Schafer and Brower's data.

TABLE V Responses given for identification of esters

Chemical Class to which esters* were ascribed	% of total response
Ester	64
Ketone	13
Aldehyde	6
Alcohol	5
Ether	4
Amine	3
Carboxylic acid	1
Hydrocarbon	1
Sulphur compound	0
Halide	0
Phenol	0
Others	3

* the esters used by Schafer and Brower were iso-pentyl butyrate, methyl benzoate and ethyl cinnamate.

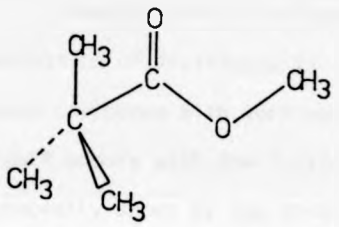
It can be seen that Ketone was the chemical class to which esters were most often wrongly ascribed. The reverse was also true and ketones were most often wrongly identified as esters.

Some ketones, such as undecan-6-one, are reported as being fruity (von Braun *et al*, 1929), and so must be able to bind to olfactory receptors in a similar way to esters.

Brower and Schafer, (1975), in another study, found that it was possible to mask recognisable functional groups by steric hindrance, as has been found with the ester function here.

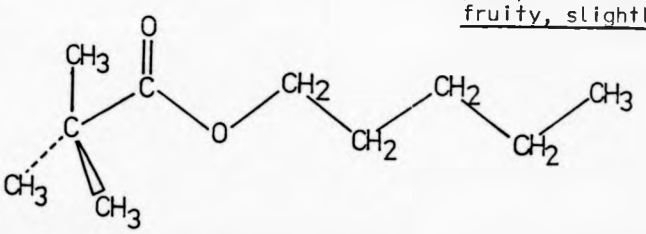
Esters such as pentyl tri-methylacetate and t-butyl pentanoate, with a long side chain as well as a bulky side group, retain a fruity quality despite steric hindrance, Figure (29).

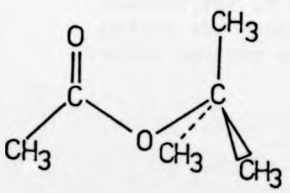
FIGURE (29)



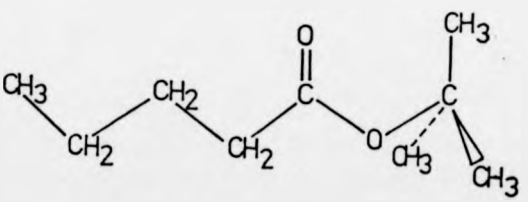
methyl tri-methylacetate
minty

pentyl tri-methylacetate
fruity, slightly minty





t-butylacetate
minty



t-butyl pentanoate
minty, fruity, earthy

Comparison between sterically hindered esters with and without long side chains.

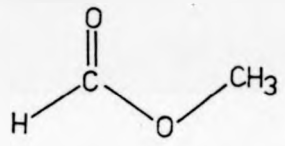
This implies that a receptor site that recognises esters as fruity must be able to bind the alkyl groups as well as the carbonyl function so that a reduction in binding due to steric hindrance of the carbonyl group can be compensated for by the binding of a long alkyl side-chain.

Recognition of the alkyl side-chains as part of the determination of fruitiness is evinced by the odour characteristics of those compounds with very small side-groups which tend to have ethereal* odours with the fruity quality weak if present at all. This is especially shown by the formates with methyl, propyl and iso-propyl formate being reported only as ethereal, see Figure (30).

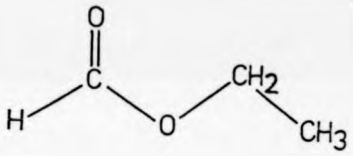
* The odour descriptions from Arctander, (1969), seemed to include 'ethereal' used rather indiscriminately. However for at least some of these low molecular weight esters the description 'ethereal' was obtained from other sources as well.

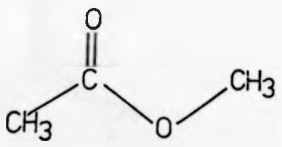
FIGURE (30)

methyl formate
ethereal

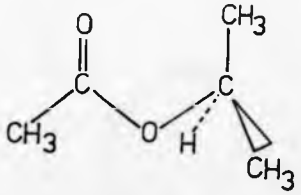


ethyl formate
fruity, ethereal, pungent, rum





methylacetate
fruity, ethereal, pungent

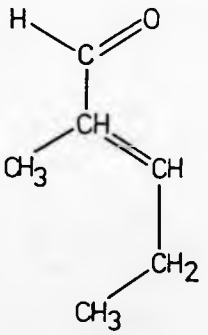


iso-propyl formate
ethereal

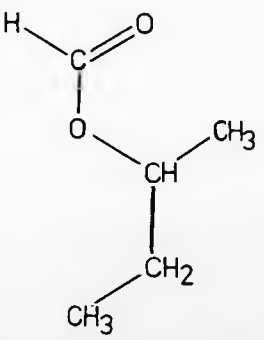
Esters with small alkyl groups tend to have ethereal rather than fruity odours showing that the alkyl groups are important determinants of the fruity odour of esters.

The discovery that sec-butyl formate smells mainly of bitter almonds was surprising at first, but, as shown in Figure (31), it can assume a configuration iso-steric with compounds described by Boelens and Heydel, (1973), in their study of the bitter almond odour. A trace of bitter almonds could also be discerned in the odour of pentyl formate.

FIGURE (31)



2-methyl pent-2-enal
bitter almonds



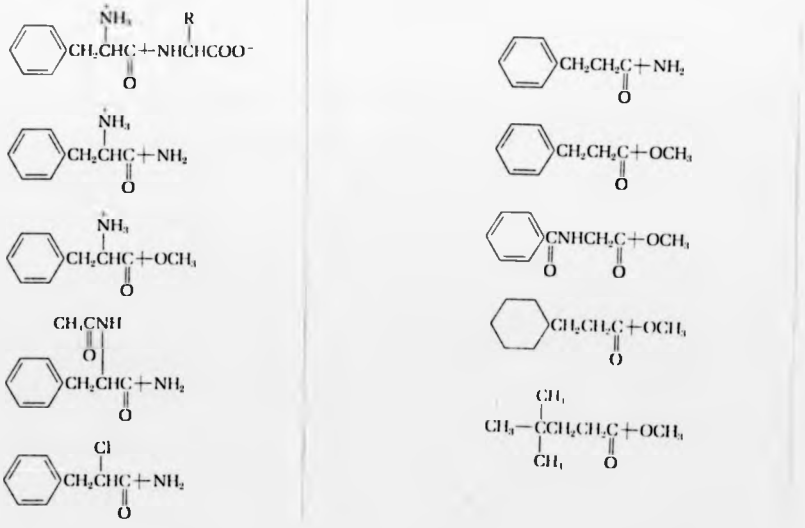
sec-butyl formate
bitter almonds, ethereal, fruity

sec-butyl formate drawn iso-steric with 2-methyl pent-2-enal, a bitter almond compound from Boelens and Heydel, (1973).

An analogy may be drawn between the postulated binding of the fruity-smelling esters to an olfactory receptor site and the binding of substrates to peptidases such as pepsin and chymotrypsin which have relatively broad specificities.

Taking chymotrypsin as an example, it has been found to hydrolyse a range of acyl compounds including esters. Figure (32), taken from Lehninger, (1970), shows some of the compounds that can act as substrates for it. These all have bulky hydrophobic side-groups which bind to a hydrophobic zone in the active site of the enzyme whilst the carbonyl group interacts with a nucleophilic serine residue.

FIGURE (32)



Compounds bound and hydrolysed by chymotrypsin.
From Lehninger, (1970).

One can postulate from the example of chymotrypsin that an olfactory receptor site binding esters might have two hydrophobic zones on either side of a nucleophilic amino acid residue that would bind to the carbonyl group, (the nucleophilic amino acid might be a serine, a cysteine or a histidine residue). Such a binding site would explain the observed odour qualities of the fruity-smelling esters.

In conclusion, it is interesting to note that Duchamp et al, (1974), in studies on single cell reponses in frog olfactory epithelium, found several units exclusively responsive to iso-pentyl acetate, the only fruity odorant in their study.

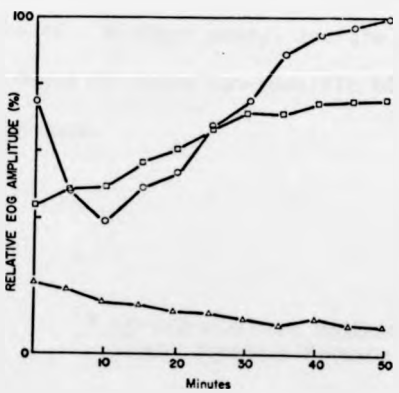
CHAPTER 4 VAPOUR PHASE LABELLING EXPERIMENTS

4.1 Introduction - Chemical Modification Experiments in Olfaction

The first use of chemical modification for studying the mechanisms of olfactory discrimination * was reported by Getchell, (1971), and Getchell and Gesteland, (1972). They used a group-specific reagent, N-ethyl maleimide, (NEM), which primarily reacts with the sulphydryl groups of proteins. Dissolved in Ringer's solution and applied to the olfactory epithelium of Rana pipiens, NEM caused a general, irreversible reduction in the epithelial and bulbar responses to odorants.

A 4mM solution of NEM applied to the olfactory epithelium for 2 minutes had the effect of reducing the peak amplitude of the EOG to ethyl butyrate by 77%. Specific protection of the EOG response was obtained by applying a 12.5mM solution of ethyl butyrate to the epithelium before, during, and after treatment with NEM. There was a 24% reduction in the EOG peak amplitude with protection versus a 77% reduction without protection, see Figure (33)

FIGURE (33)



Specific protection of the EOG response to ethyl butyrate against the effects of NEM, (Getchell and Gesteland, 1972). Ordinate: EOG peak amplitude as % of value before treatment. Abscissa: Time after treatment, (minutes).
Δ—Δ Effects of NEM alone - inhibition of EOG response.
□—□ Effects of NEM in the presence of ethyl butyrate - the response to ethyl butyrate is protected.
O—O Effects of ethyl butyrate alone.

* for a general review of chemical modification see Singer, (1967).

The protection afforded by ethyl butyrate was shown to be extended to methyl butyrate, but not to dissimilar compounds such as cis-1,2-dichloroethylene.

Getchell and Gesteland, (1972), interpreted their results as evidence for the existence of specialised, proteinaceous olfactory receptor sites.

Using another sulphydryl reagent, mersalyl*, Menevse, (1977), performed similar experiments on Rana temporaria and obtained similar results. Mersalyl is not membrane permeable and can therefore only react with proteins on the outer surface of intact cells, so Menevse's (1977) experiments can be taken as evidence that olfactory receptor sites are located on the exterior of the receptor cell membrane. (NEM is penetrant and will therefore react with both intra and extracellular proteins).

Mersalyl was found to cause rapid, irreversible, concentration-dependent inhibition of the EOG response. In protection experiments analogous to those of Getchell and Gesteland, (1972), Menevse, (1977), used 1.0mM pentylacetate to block the effects of 0.1mM mersalyl. The differential protection afforded to various odorants is shown in Table VI. Without pentyl acetate present, a 0.1mM mersalyl solution was found to cause non-specific 60 - 80% reduction in EOG peak amplitudes.

* (3-((2-(Carboxy methoxy)-benzoyl)amino)-2-methoxy propyl)hydroxy Mercury

TABLE VI Protection by pentyl acetate against the effects of mersalyl

Odorant	Odour description	Reduction in EOG Peak Amplitude (% Original Value ± S.E.M.)
Pentyl acetate	Fruity	14 ± 4
Butyl acetate	Fruity	22 ± 7
1,8-cineole	Camphoraceous	23 ± 11
Butyric acid	Sweaty	26 ± 10
Phenylacetaldehyde	Green, floral	28 ± 2
Butanol	Fusel oil	50 ± 6
Nitrobenzene	Bitter almond	52 ± 10
Benzyl acetate	Fruity, jasmin	62 ± 10
beta-Ionone	Violets	65 ± 8
Linalyl acetate	Floral, fruity	72 ± 11
Reductions expected with no protection		60 - 80

Taken from the results of Menevse, (1977).

So although pentyl acetate gave most protection to its own receptors and to those of the very similar butyl acetate, there was no simple relationship, (in terms of structure and odour quality), between pentyl acetate and the other odorants to account for the relative degrees of protection afforded them. Getchell and Gesteland's, (1972), apparently more straight forward results were based on a smaller survey.

The chemical modification studies described above relate to group-specific labelling, the kinetic scheme for which is given in Equation (1).

Equation (1) Group-specific labelling

$$L + R \longrightarrow L\text{-}R$$

where L = chemical modification reagent

R = receptor

The chemical modification experiments which will be described in the results section of this chapter are concerned with affinity labelling, which has been reviewed by Singer, (1970), and see also Jakoby and Wilchek, (1977). The kinetic scheme for this type of labelling is given in Equation (2).

Equation (2) Affinity labelling

$$L + R \underset{-k_1}{\overset{k_1}{\rightleftharpoons}} L.R \xrightarrow{k_2} L\text{-}R$$

where L.R = reversible complex between ligand and receptor

L-R = ligand covalently attached to receptor

In affinity labelling chemical modification experiments the label combines specifically and reversibly with the receptor, then,

by virtue of a reactive group incorporated into it, the label forms a covalent bond to a suitable disposed amino acid residue in the binding site. The labelling is made specific because the initial reversible binding of the label to the receptor in question increases the local concentration of the label in the binding site compared to the concentration in free solution, (Wofsy et al, 1962), and because the micro-environment within the binding site may cause amino acid residues within it to have heightened reactivity.

Affinity labelling, as a manner of attaining specific, in situ labelling, may prove to be a key technique in the isolation and identification of olfactory receptor proteins. In situ labelling would be desirable because the activity of the olfactory receptors is expressed in the binding of odorants and the characteristics of this binding could well be drastically altered in the course of isolation. In addition, by labelling the receptors in the intact system, the degree of labelling can be monitored indirectly through physiological parameters such as the EOG or behavioural responses. (See Singer et al, 1973).

Menevse et al, (1976, 1977,a), have shown that odorants incorporating a reactive group applied to frog olfactory epithelium in the vapour phase can selectively, and irreversibly inhibit their own EOG responses, presumably by blocking their own receptors. Figure (34) shows an experiment where 4-chloro 7-nitro benzofurazan, (Nbf-chloride), which will covalently attach itself to amino and sulphydryl groups, (Ghosh and Whitehouse, 1968, and Fager et al, 1973), caused a specific decrease in the EOG peak amplitude to itself, but caused a much smaller decrease in the EOG's to 1,8-cineole

and pentyl acetate. Figure (34,b) shows the experiment replotted to indicate the total time of exposure of the epithelium to Nbf-chloride (this is the way experiments in the results section will be plotted).

Menevse et al, (1977,a), also used NEM, 1-fluoro 2,4-dinitro benzene and benzyl chloride to provide specific, vapour-phase chemical modification. The selectivity of the labelling was not demonstrable when the reagents were applied to the olfactory epithelium in the liquid phase.

The affinity labelling experiments reported here have been performed using vapour-phase alpha-haloacetates, (these are alkylating agents).

FIGURE (34,a)

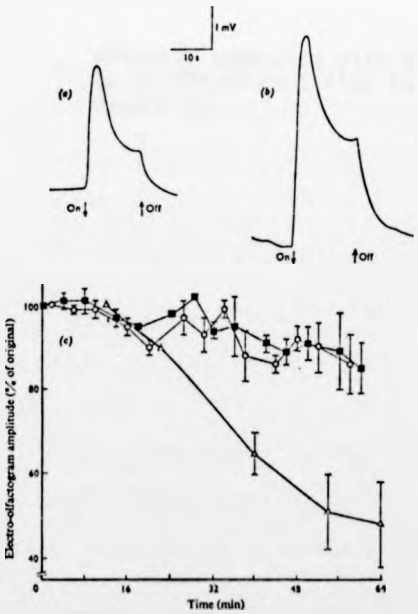
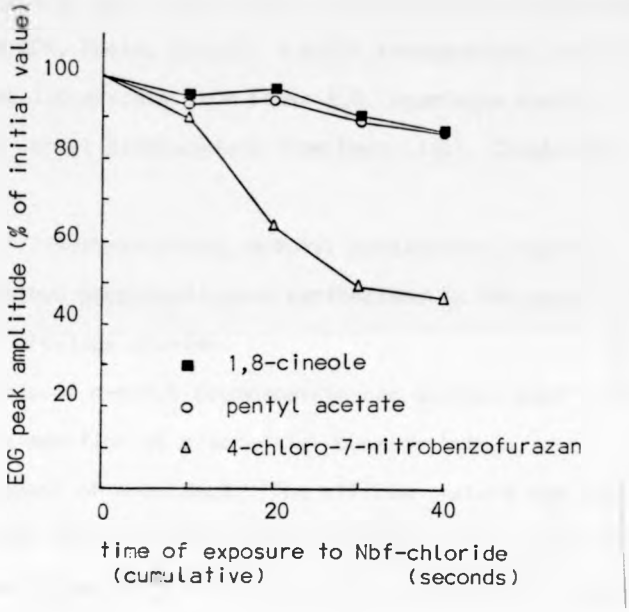


Fig. 1. *Specific labelling of receptors for the odorant 4-chloro-7-nitrobenzofurazan*
(a) Typical electro-olfactogram for this odorant; (b) typical electro-olfactogram for *n*-amyl acetate. The points in (c) are means ± 1 S.E. of three experiments ■, 1,8-Cineole; ○, *n*-amyl acetate; △, 4-chloro-7-nitrobenzofurazan. The vapour pulses were of 10 s duration, with an interval of 2 min between each. The general experimental methods were as described by Getchell & Gesteland (1972).

Vapour phase affinity labelling of olfactory receptors by Nbf-chloride from Menevse et al, (1977,a)

FIGURE (34.b)



Affinity labelling with Nbf-chloride, same experiment as in (34,a) replotted to show time-course of labelling.

4.2 Materials and Methods

Ethyl chloroacetate was obtained from the Aldrich Chemical Company Ltd., Gillingham, Dorset; ethyl acetate, pentyl acetate and 1,8-cineole from BDH, Poole, Dorset; t-butyl bromoacetate, bromoacetyl bromide and ethyl iodoacetate from Fluka A.G. Chemische Fabrik, Switzerland; and ethyl bromoacetate from Koch Light, Colnbrook, Buckinghamshire.

Ethyl tri-methylacetate, n-butyl propionate, t-butyl propionate, and ethyl propionate were synthesised in the manner described in the previous chapter.

Synthesis of n-butyl bromoacetate was accomplished by the careful, dropwise addition of bromoacetyl bromide into a stoichiometric amount of n-butanol. The stirred mixture was left to cool for 30 minutes before being vacuum distilled. The product was identified by the comparison of its ^{1}H N.M.R. spectrum with that of n-butyl acetate, all resonances being comparable excepting the peak associated with the alpha-hydrogens which was shifted downfield by 1.93 Tau in the bromoacetate. The n-butyl bromoacetate was twice redistilled under vacuum until 96+% pure by G.L.C. (for conditions see section 2.5 (iv)).

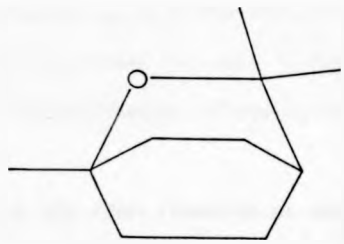
The other odorants and affinity labels used were pure as determined by G.L.C. analysis, (section 2.5 (iv)), except for ethyl iodoacetate, two samples of which were obtained, both being only about 50% pure.

The experiments were performed on an *in vivo* frog preparation at room temperature, ($22 \pm 2^{o}C$). Only frogs giving stable EOG responses were used. The recording electrode was consistently placed on the peak

of the olfactory eminence. Vapour phase odorant concentrations were adjusted to give EOG's in the 1.5 to 6.0mV range. Fatigue of the tissue (adaptation) was avoided by allowing a period of at least 3 minutes between stimulus presentations and by keeping the stimulus duration to 10 seconds or less.

The camphoraceous odorant 1,8-cineole was included in all experiments to give a reference point by which they may be compared. Its structure (Figure (35)), and odour characteristics are unrelated to those of other compounds used.

FIGURE (35)



1,8-cineole CAMPHORACEOUS

The affinity labelling reagents used in this study are also odorants, so they will be referred to as affinity labelling odorants.

Experimental protocol

In all experiments, prior to exposure of the epithelium to the affinity labelling odorant, at least 3 EOG's were obtained from each of the other odorants used. The peak amplitudes of these were averaged to give the (100%) value against which subsequent responses were compared.

The channel carrying the affinity labelling odorant was then switched on for a set period of time, (usually 15 seconds), and the peak height of the resultant EOG gave the 100% value for this compound.

After a 5 - 10 minute rest EOG's were elicited to each of the odorants in turn finishing up with the affinity labelling odorant, the presentation of which comprised the next 'aliquot' of labelling. These EOG's indicated the responsiveness of the epithelium after the first period of labelling.

The sequence was then repeated as many times as necessary.

Results are presented graphically with the ordinate giving the EOG amplitudes as a percentage of the starting values and the abscissa giving the total time that the epithelium was exposed to the affinity labelling odorant. All points represent the mean from experiments on separate frogs $\pm$ 1 standard deviation. Plotted in this way the graphs show the time course of labelling.

The vapour phase concentrations of most of the odorants used have been determined using a gas sensor (TGS 812 gas sensor, from Watford Electronics Ltd., 33 Cardiff Road, Watford, Hertfordshire). The settings on the olfactometer governing odorant flow rates were

kept constant throughout the series of experiments with vapour phase odorant concentrations being measured on several separate occasions. The determinations are given in Table VII. I am grateful to Mr. K. C. Persaud for the calibration curves.

TABLE VII

Odorant	Vapour Phase ** Concentration (microMoles/ litre air)	± 1 S.D.	Number of Determinations
n-butyl propionate	0.70	± 0.30	5
t-butyl propionate	0.78	± 0.32	6
1,8-cineole	0.06	± 0.02	7
ethyl acetate	15	-	1
ethyl bromoacetate	1.58	± 0.15	6
ethyl tri-methyl acetate	0.93	-	1
ethyl propionate	8.7	± 2.2	8
pentyl acetate	0.73*	-	-

* calculated
** as presented to epithelium

Determination of vapour phase concentrations of odorants as used in the experiments reported in this chapter. At these concentrations the EOG responses elicited had peak amplitudes of between -1.5 and -6.0 mV.

Note:

In the following results section the abscissae of the graphs are given as 'cumulative time of labelling'. Since a labelling mechanism is what the experiments are designed to show, these abscissae should be given as 'time course of inhibition of EOG responses'.

4.3 Results

The results presented here are from a series of experiments, the aim of which is to optimise conditions for the affinity labelling of olfactory receptors so that, ultimately, they may be isolated and characterised.

4.3 i) Comparative effects on EOG responses of ethyl chloro acetate, ethyl bromoacetate and ethyl iodoacetate

All three of these compounds have fruity, apple-like odours, similar to that of ethyl propionate, and will alkylate nucleophilic amino acid residues. The other odorants used were 1,8-cineole, (camphoraceous) and pentyl acetate, (fruity, bananas).

Conditions were kept constant between the three experiments. The settings on the olfactometer were adjusted so that the flow rates of air through the U-tubes containing the affinity odorants were identical.

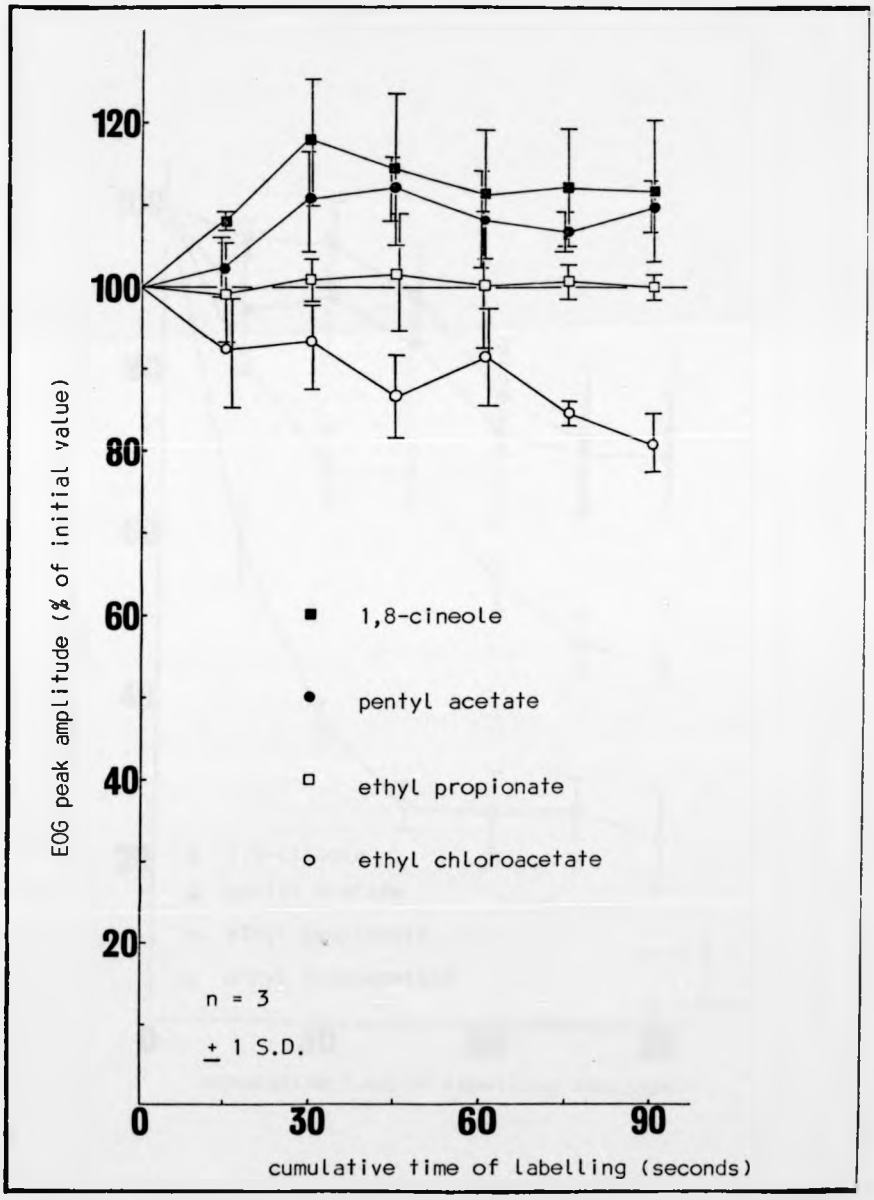
Figure (36) on page 91 shows the time course of labelling by ethyl chloroacetate.

Figure (37) on page 92 shows the time course of labelling by ethyl bromoacetate.

Figure (38) on page 93 shows the time course of labelling by ethyl iodoacetate.

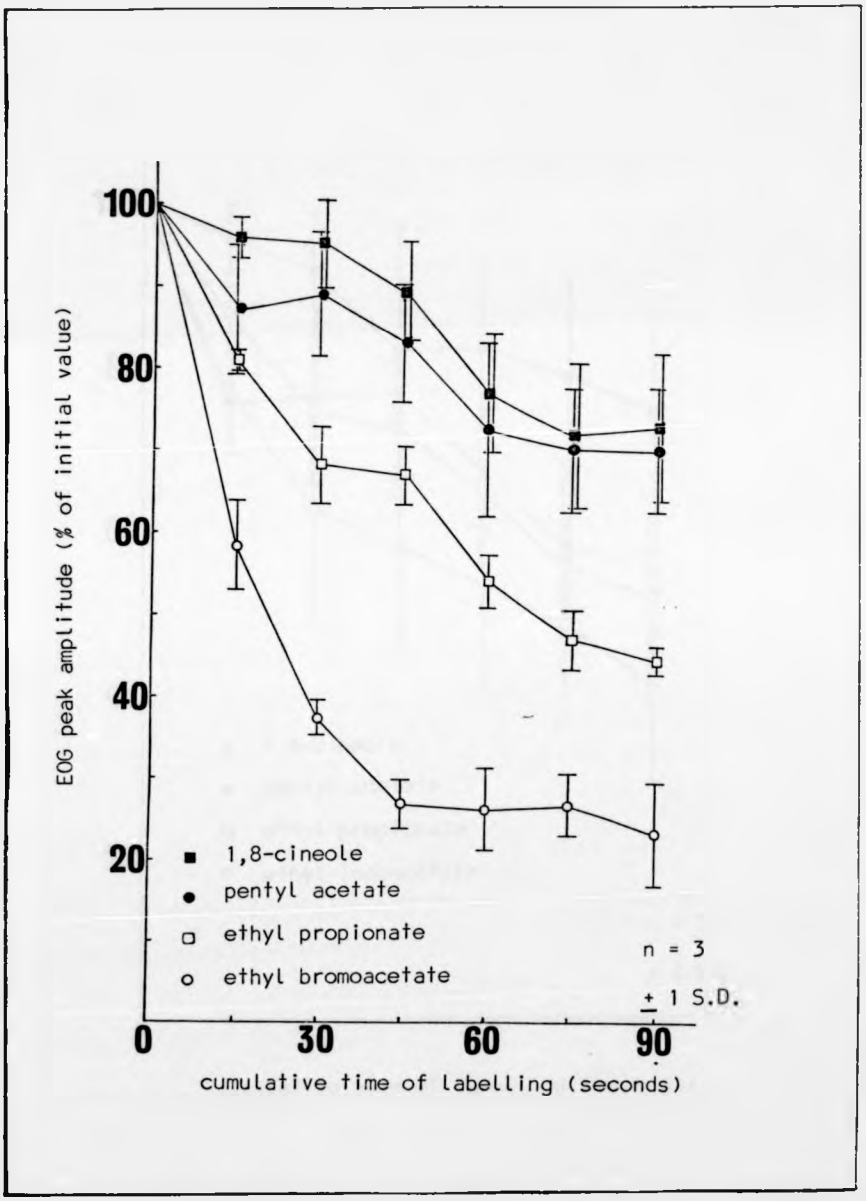
The number of experimental animals used (n) is given for each figure.

FIGURE (36)



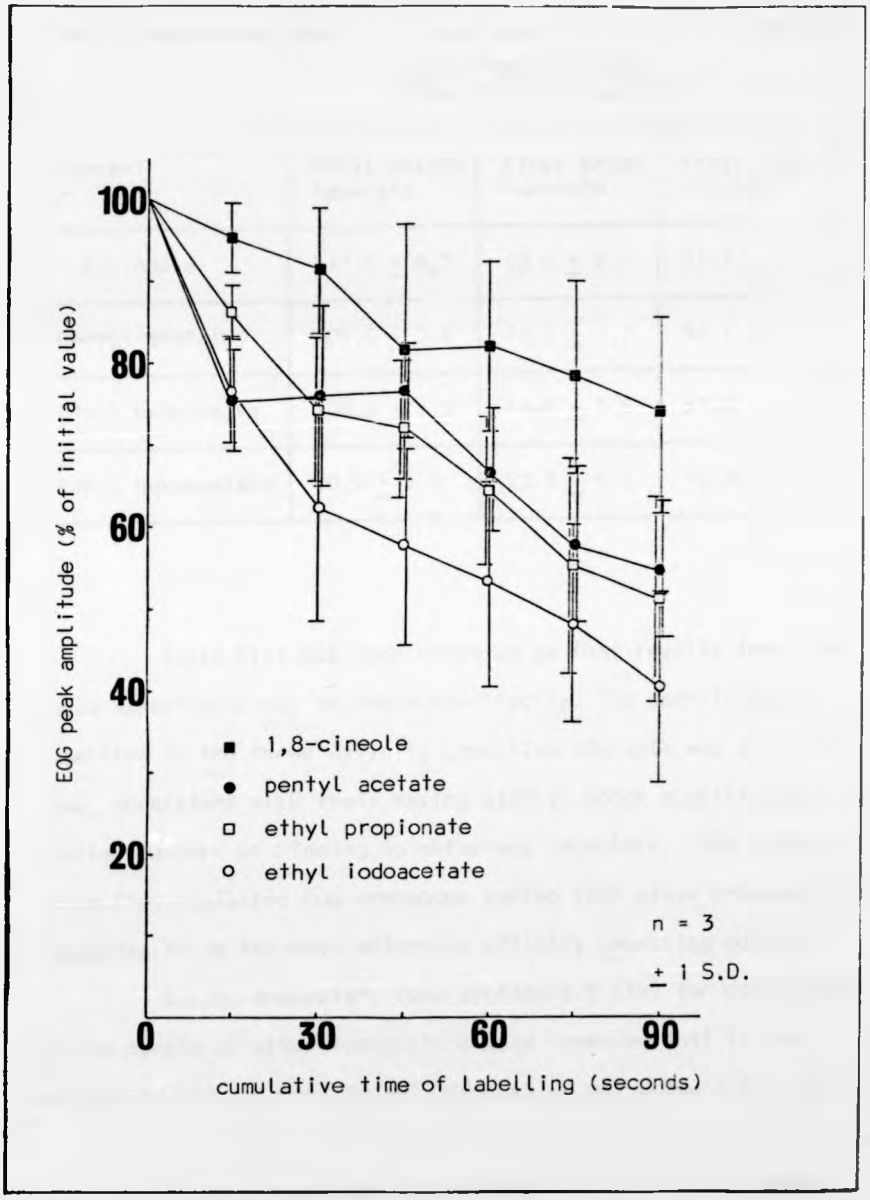
Time course of affinity labelling with ethyl chloroacetate.

FIGURE (37)



Time course of affinity labelling with ethyl bromoacetate

FIGURE (38)



Time course of affinity labelling with ethyl iodoacetate

TABLE VIII Comparative Inhibition of EOG Resonses by Ethyl haloacetates, (n = 3, ± 1 S.D.)

	% EOG Peak Amplitude After 90 Secs Labelling		
Odorant	Ethyl chloro -acetate	Ethyl bromo -acetate	Ethyl iodo -acetate
1,8-cineole	111.6 ± 8.7	73.0 ± 9.3	73.7 ± 12.1
Pentyl acetate	109.6 ± 3.4	70.2 ± 7.7	54.7 ± 8.4
Ethyl propionate	100.0 ± 1.5	44.6 ± 1.6	51.0 ± 12.6
Ethyl haloacetate	80.9 ± 3.6	23.3 ± 6.4	40.3 ± 11.7

Table VIII has been drawn up so that results from the above experiments may be compared directly. The specificity of labelling by the three affinity labelling odorants was much the same, consistent with their having similar odour qualities and a similar pattern of binding to olfactory receptors. The extent to which they inhibited EOG responses varied with ethyl bromoacetate appearing to be the most effective affinity labelling odorant.

G.L.C. analysis*, (see section 2.5 (iv) for conditions), of the sample of ethyl iodoacetate used revealed that it had decomposed and was only ca.50% pure, which may account for the

* All compounds were analysed after they were used to check for decomposition.

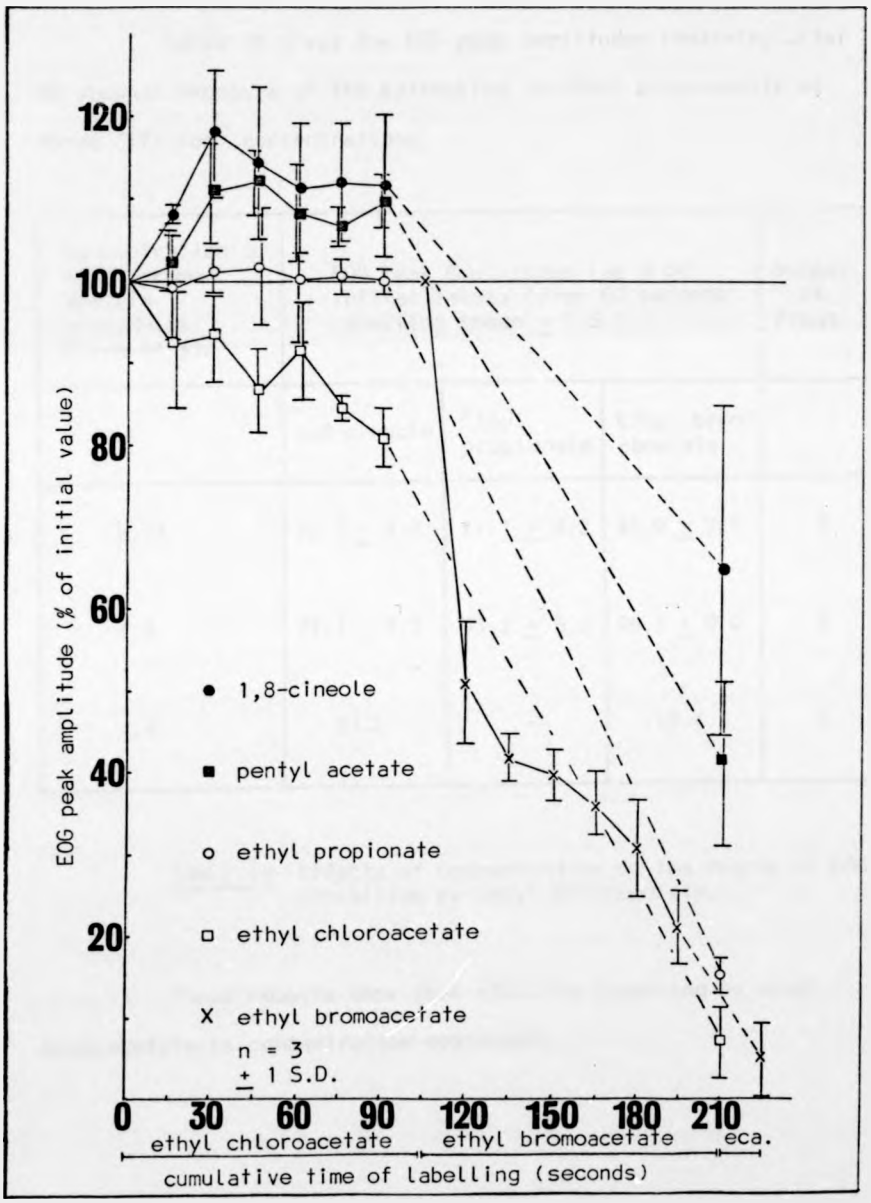
degree of labelling by ethyl iodoacetate being less than that of ethyl bromoacetate.

Since ethyl bromoacetate combined specificity with a high degree of labelling, and proved to be stable when kept in a U-tube, at room temperature, it was chosen for use in further experiments.

Figure (36) showed the effects of treating the epithelium with ethyl chloroacetate, the response to which was reduced by a relatively small amount, whilst the peak amplitude of the EOG's elicited by 1,8-cineole and pentyl acetate were actually increased. The experiment was continued, (see Figure (39) overleaf). After treatment with ethyl chloroacetate the epithelium was exposed to ethyl bromoacetate for a total of 90 seconds. This resulted in a reduction in EOG peak amplitudes to all the odorants used, with the responses to ethyl chloroacetate and ethyl bromoacetate almost being abolished. This confirms that ethyl bromoacetate is much more effective than ethyl chloroacetate.

Figure (39). Time course of labelling by ethyl chloroacetate followed by ethyl bromoacetate.

FIGURE (39)



Affinity labelling with ethyl chloroacetate
followed by ethyl bromoacetate

4.3 ii) Concentration dependence of affinity labelling by ethyl bromoacetate

Table IX gives the EOG peak amplitudes remaining after 60 seconds exposure of the epithelium to ethyl bromoacetate at three different concentrations.

Concentration of Ethyl bromo-acetate micro'moles/ litre of air	EOG Peak Amplitudes (as % of Initial Value) After 60 seconds Labelling (mean ± 1 S.D.)			Number of Frogs
	1,8-cineole	Ethyl propionate	Ethyl bromo -acetate	
0.34	79.7 ± 2.7	71.1 ± 4.2	45.9 ± 7.7	3
1.6	77.1 ± 7.2	53.2 ± 3.2	26.3 ± 5.0	3
7.4	53.2	-	15.4	1

TABLE IX Effects of Concentration on the Degree of EOG Inhibition by Ethyl bromoacetate.

These results show that affinity labelling by ethyl bromoacetate is concentration dependent.

4.3 iii) Protection experiments

An important criterion for establishing that affinity labelling is being observed is the demonstration of protection, (Singer, 1970). In the presence of a ligand for the binding site in question the rate of its specific inactivation should be reduced.

Figures (40), (41), (42), show that such an effect can be demonstrated with affinity labelling by ethyl bromoacetate.

Figure (40) shows the time course of inhibition of the EOG responses to 1,8-cineole and ethyl propionate by ethyl bromoacetate at a concentration of 1.6 microMoles/litre of air, (i.e. Figure (36) with pentyl acetate not shown for the sake of clarity).

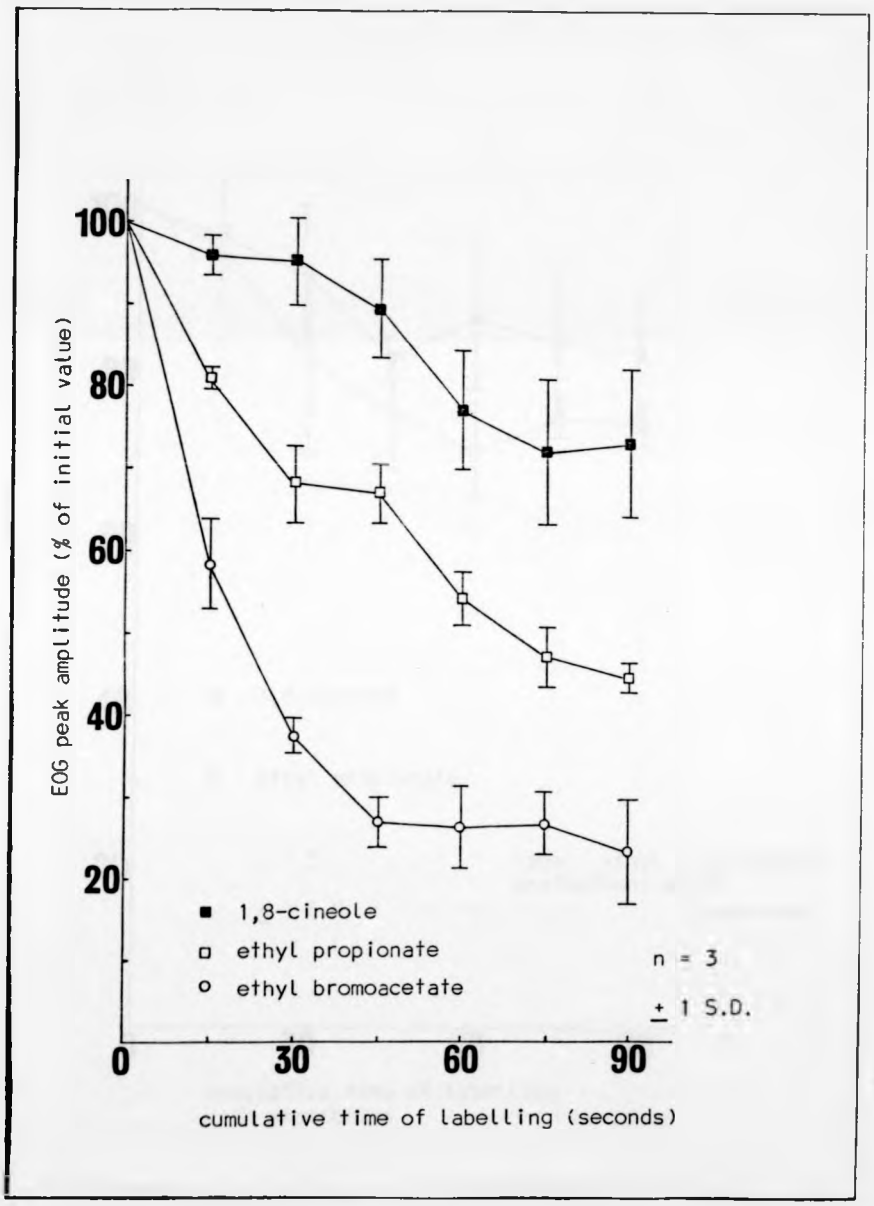
Figure (41) shows the time course of inhibition of the EOG responses to 1,8-cineole and ethyl propionate when ethyl propionate, at a concentration of 40.9 microMoles/litre of air, was applied to the epithelium before, during, and after treatment with ethyl bromoacetate at the same concentration as above. The protocol followed was to switch on the channel carrying the ethyl propionate for protection 10 seconds before each 15 second 'aliquot' of labelling by ethyl bromoacetate, and to turn it off 15 seconds afterwards. Independent channels were used for carrying ethyl propionate for protection, and ethyl propionate as an odorant which, as previously, was used at 8.7 microMoles/litre of air.

In the presence of such a high concentration of ethyl propionate, the EOG response to ethyl bromoacetate could not be

measured. A ten minute rest for recovery from adaptation was allowed after treatment.

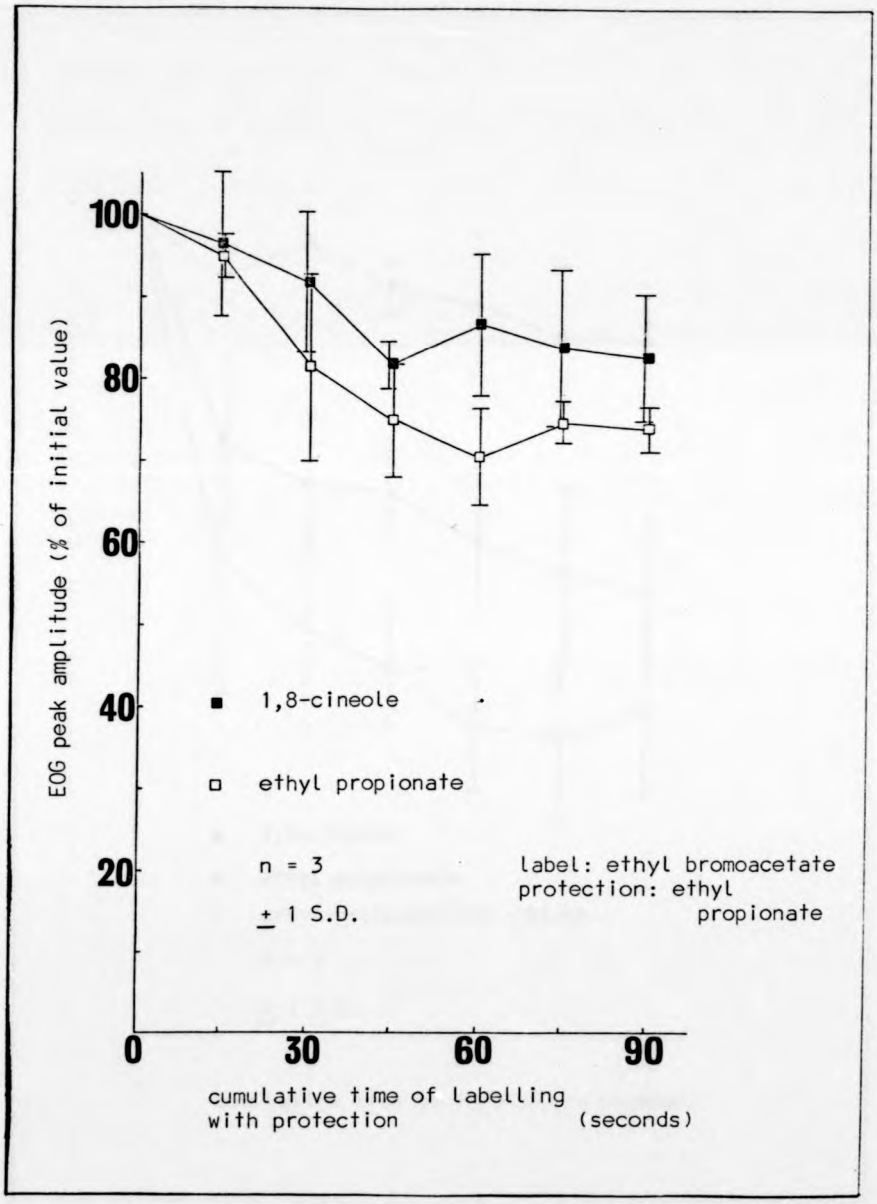
Figure (42) shows a control experiment for the above treatment. Since the air carrying the ethyl propionate for protection might have acted to dilute the affinity labelling odorant and thus have been the cause of the reduced inhibition, an analogous experiment was carried out to the one shown in Figure (41) with an equal volume of pure air replacing the air stream which carried the ethyl propionate for protection, (i.e. the U-tube containing the ethyl propionate was by-passed). In this case the EOG to ethyl bromoacetate could be measured. Again ten minutes was allowed for recovery from adaptation after treatment.

FIGURE (40)



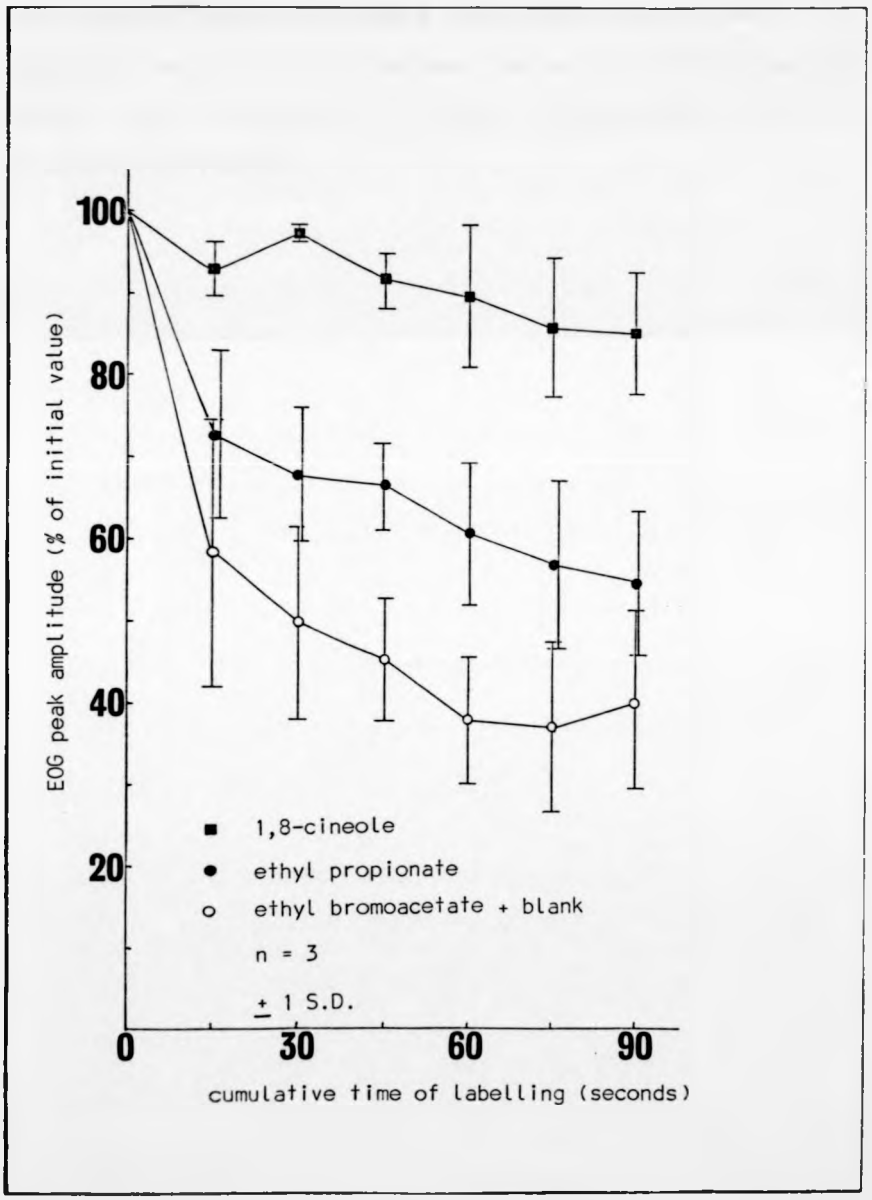
Time course of labelling without protection
with ethyl bromoacetate used as affinity labelling odorant

FIGURE (41)



Time course of labelling with ethyl bromoacetate
EOG's to ethyl propionate protected

FIGURE (42)



Control for protection experiment

A comparison of these three figures clearly shows that inhibition of the EOG response to ethyl propionate was less, both relatively and absolutely, when a high concentration of ethyl propionate was presented at the same time as the affinity labelling odorant, ethyl bromoacetate. This can be taken as being indicative of specific protection.

4.3 iv) Specificity of inhibition of EOG responses by ethyl bromoacetate

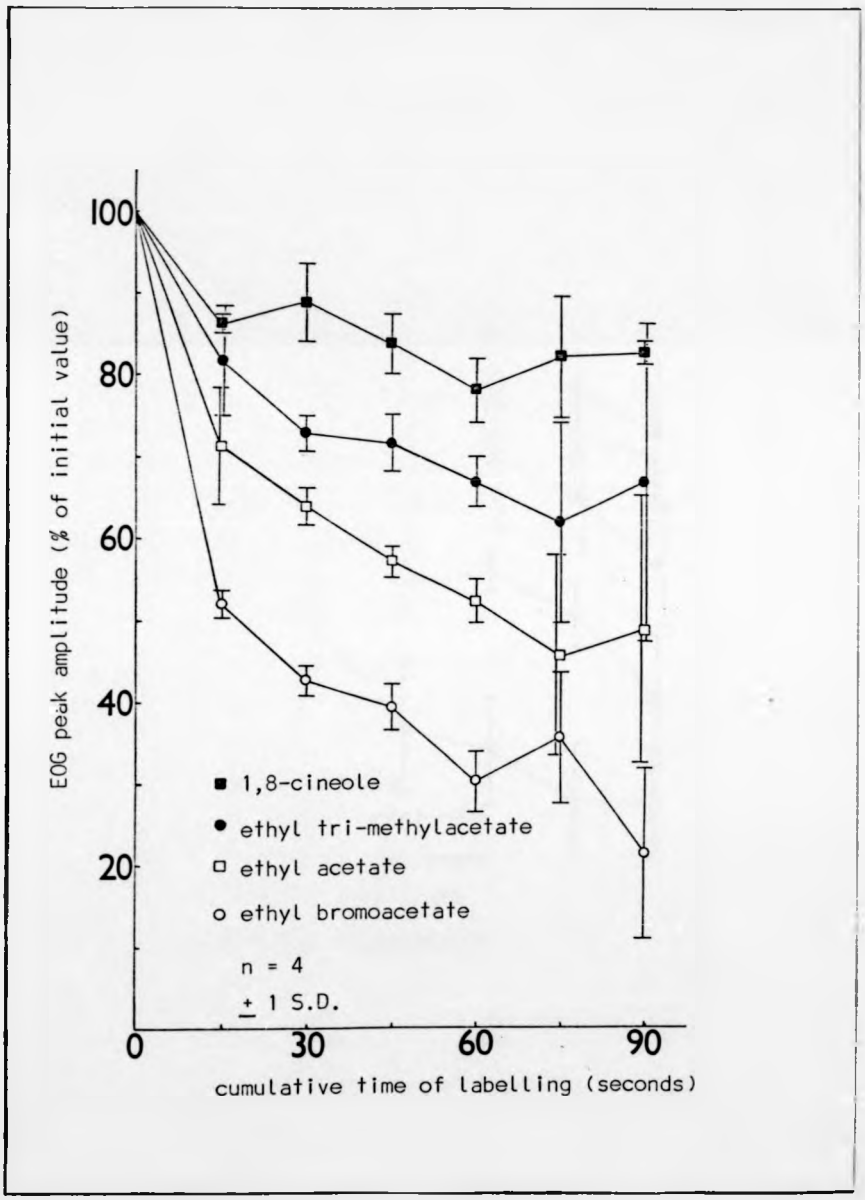
To gain information on the specificity of labelling by ethyl bromoacetate it was necessary to ascertain its effects on the EOG responses to several odorants. The experiment shown in Figure (37) demonstrated the effects of ethyl bromoacetate on the EOG's to 1,8-cineole, pentyl acetate, ethyl propionate and to itself. Esters described in Table III, page 67, were chosen as additional odorants for study since the structure-activity relationship work can then give a frame-work for interpreting the results from labelling experiments.

Conditions were kept the same as for the experiment shown in Figure (37). Esters, (RCOOR'), were chosen such that both R and R' groups were varied, and so that different odour qualities were represented.

Figure (43) shows the time course of the effects of labelling by ethyl bromoacetate on EOG's to 1,8-cineole, ethyl tri-methylacetate, ethyl acetate, and itself, (i.e. varying R).

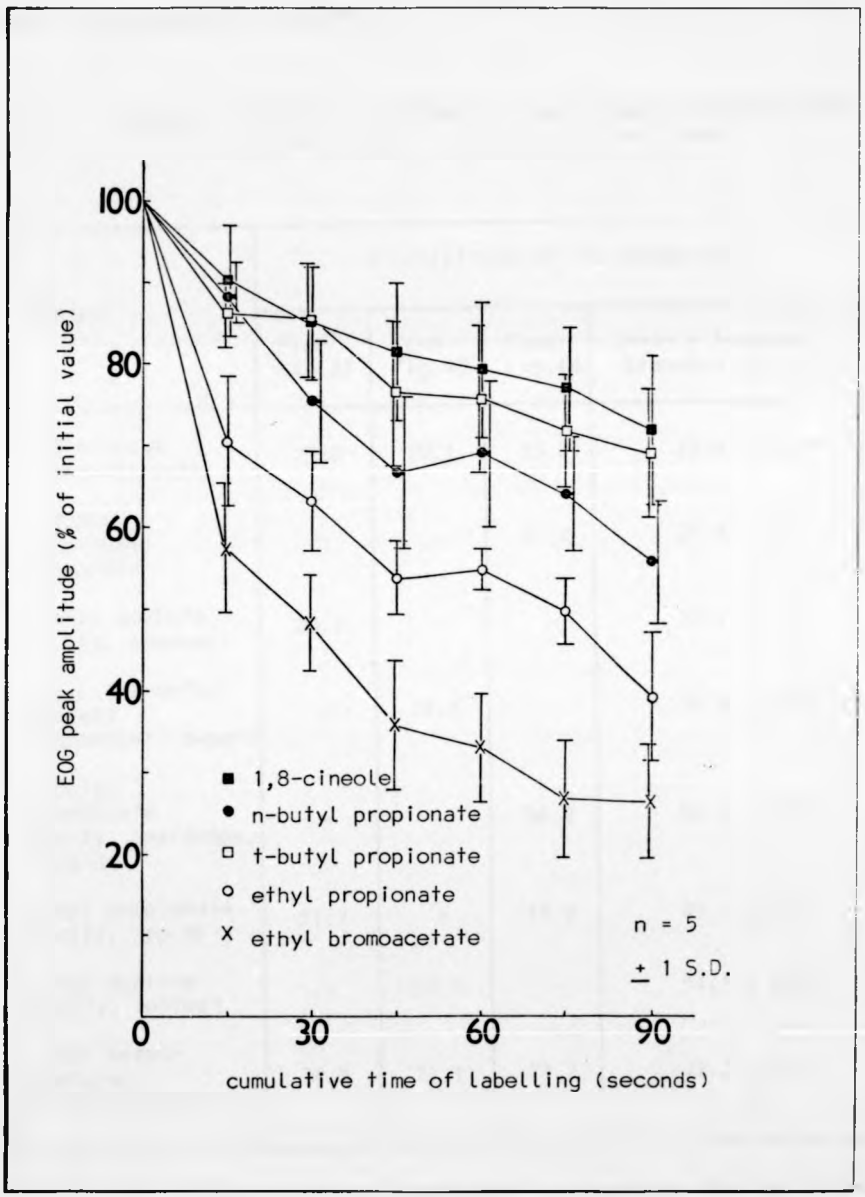
Figure (44) shows the time course of the effects of labelling by ethyl bromoacetate on EOG's to 1,8-cineole, t-butyl propionate, n-butyl propionate, ethyl propionate, and to itself, (i.e. varying R').

FIGURE (43)



Time course of affinity labelling with ethyl bromoacetate; alkanoate group of esters varied

FIGURE (44)



Time course of affinity labelling with ethyl bromoacetate; alkyl group of esters varied.

The relative degrees of inhibition of the EOG responses to all the odorants used in the experiments shown in Figures (37), (43) and (44) are compared in Table X.

TABLE X Differential effects of ethyl bromoacetate on the EOG peak amplitude to several odorants.

Odorant	% Inhibition of EOG Responses*			
	From Fig.37	From Fig.43	From Fig.44	Mean ± Average Standard Deviation
1,8-cineole (Camphoraceous)	25.9	19.1	23.8	23.0 ± 8.7
t-butyl propionate (peppermint)	-	-	27.4	27.4 ± 8.0
pentyl acetate (fruity, bananas)	29.1	-	-	29.1 ± 8.5
ethyl tri-methyl acetate (peppermint, sweet)	-	34.8	-	34.8 ± 11.6
n-butyl propionate (fruity, peardrops, bananas)	-	-	36.9	36.9 ± 7.9
ethyl propionate (fruity, apples)	51.7	-	48.8	49.7 ± 3.9
ethyl acetate (fruity, apples)	-	51.1	-	51.1 ± 10.5
ethyl bromo-acetate (fruity, apples)	74.5	71.1	71.2	72.3 ± 6.5

* Values have been calculated by averaging the last three points on each curve in Figures (37), (43) and (44), so each figure represents the mean of 9 - 15 measurements.

Ethyl bromoacetate had the greatest effect on the EOG's it elicited itself, whilst having least effect on the responses to 1,8-cineole, which is the compound it is most different from in terms of structure and odour quality.

A comparison of the effects of ethyl bromoacetate on the response to esters with the same, small alkanoate group and alkyl side chains of different lengths shows that the responses were more inhibited when the alkyl side chain was small:

a)	Ethyl acetate	51.1% inhibited
	Pentyl acetate	29.1% inhibited
b)	Ethyl propionate	49.7% inhibited
	n-butyl propionate	36.9% inhibited

The responses to pentyl acetate, which had the longest side chain, were least affected.

The responses to ethyl acetate and ethyl propionate were inhibited by very similar degrees after labelling with ethyl bromoacetate. The two compounds have very similar odours.

Two esters included in these experiments were chosen because they have an odour quality different to that of the other esters. Ethyl tri-methylacetate and t-butyl propionate have a tertiary butyl group adjacent to the ester function, steric hindrance of which results in loss of the fruity odour quality normally associated with esters. Ethyl tri-methylacetate and t-butyl propionate have peppermint odours, with ethyl tri-methylacetate having a sweeter smell than that

of t-butyl propionate, which is purely minty. The t-butyl group is on a different side of the ester function in the two compounds.

The inhibition of the EOG responses to these two compounds is compared with that of their non-sterically hindered counterparts:

a) Ethyl acetate (fruity)	51.1% inhibited
Ethyl tri-methylacetate (peppermint)	34.8% inhibited
b) Ethyl propionate (fruity)	49.7% inhibited
t-butyl propionate (peppermint)	27.4% inhibited

Labelling had much less effect on the responses to the sterically hindered esters with a minty odour than on the responses to the esters with a fruity odour similar to that of ethyl bromoacetate.

4.3 v) Inhibition of EOG responses by n-butyl bromoacetate and t-butyl bromoacetate

The effects of n-butyl bromoacetate on EOG responses were examined to see if it might not prove to be a more effective affinity labelling odorant than those tried previously.* Being a larger molecule than ethyl bromoacetate it may be capable of more well-defined interactions with the olfactory receptors and therefore give labelling of greater specificity.

The effects of t-butyl bromoacetate were examined for the same reason and also because it has a different odour quality. The ethyl haloacetates have fruity, apple-like odours, n-butyl bromoacetate has a fruity, oily smell and, as would be predicted from the structure -activity relationship studies, t-butyl bromoacetate has a peppermint odour. (It is actually a much more powerful minty odorant than, for example, t-butyl acetate or t-butyl propionate). Since the labelling actions of an affinity label are directed by its specificity of binding, the different odour quality of t-butyl bromoacetate, reflected in its pattern of binding to olfactory receptor sites, should yield a different pattern of inhibition of the EOG's to that found in the experiments above.

The use of this compound as an affinity labelling odorant can therefore act as another control for the effects of ethyl bromoacetate.

The experimental conditions for labelling by n-butyl bromoacetate were the same as for ethyl bromoacetate; for t-butyl

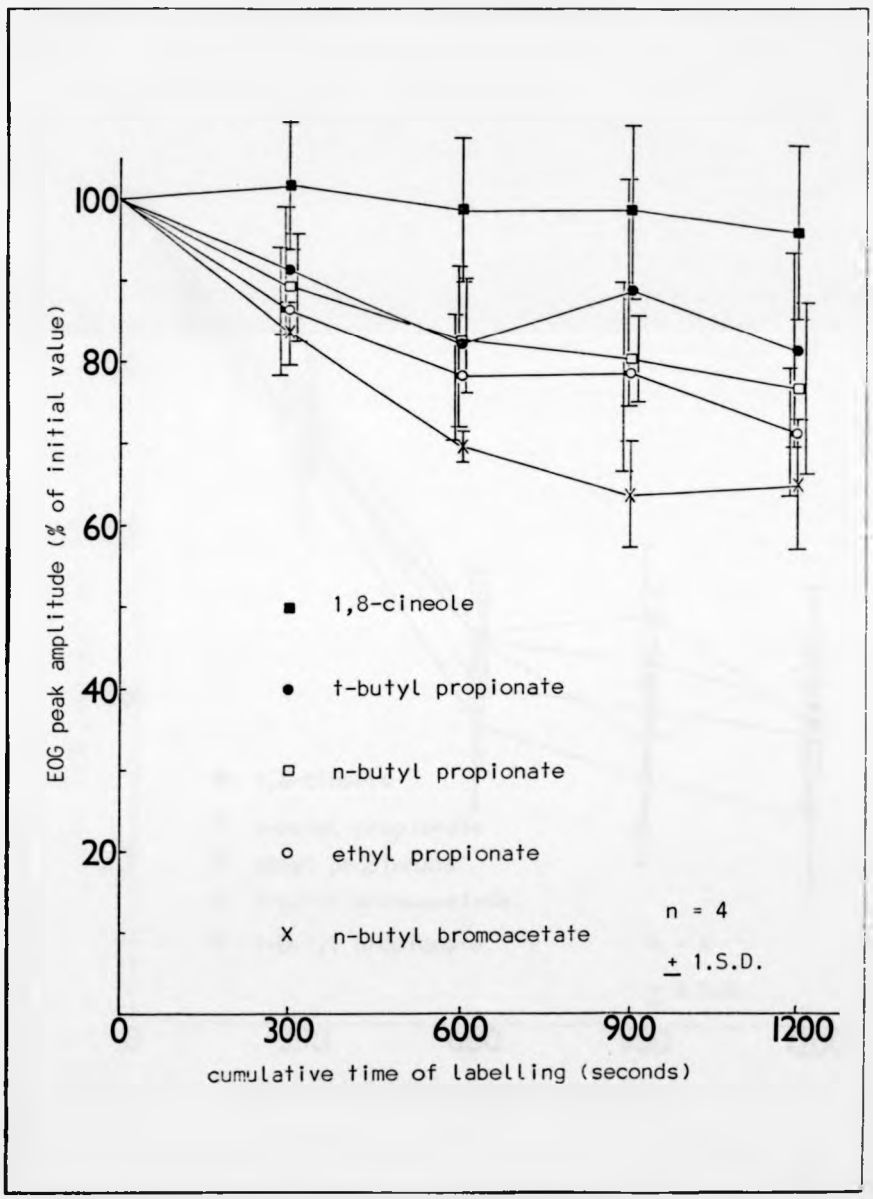
* In addition, tritiation of but-3-enol to give radiolabelled butanol followed by synthesis to yield the bromoacetate might provide a convenient route for obtaining a radioactively tagged affinity labelling odorant.

bromoacetate the flow rate of the air stream through the U-tube in which it was contained was doubled.

Figure (45) shows the time course of effects of n-butyl bromoacetate on EOG responses.

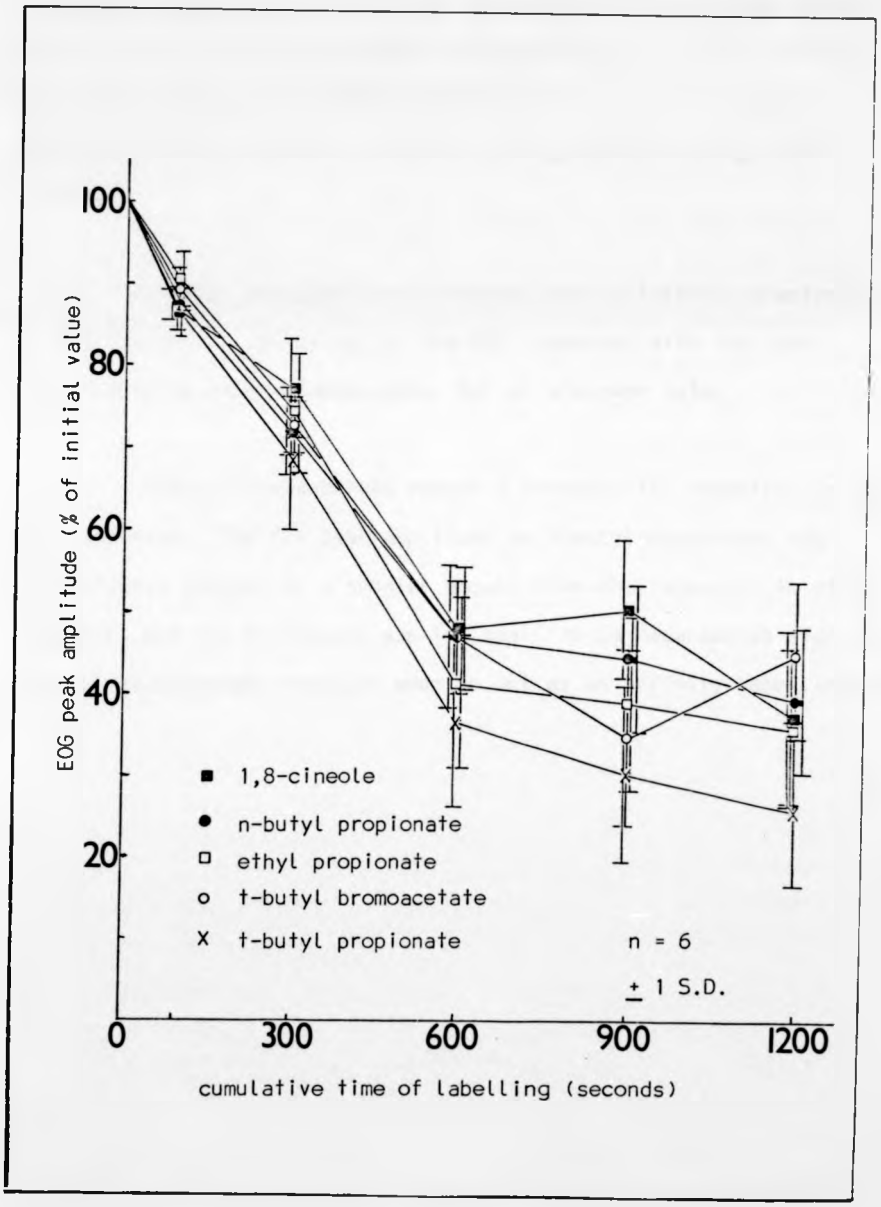
Figure (46) shows the time course of effects of t-butyl bromoacetate on EOG responses.

FIGURE (45)



Time course of labelling with n-butyl bromoacetate.

FIGURE (46)



Time course of labelling with t-butyl bromoacetate.

In two experiments the labelling by n-butyl bromoacetate (Figure (45)) was continued for a further 1200 seconds. After the total of 2400 seconds of labelling the degrees of inhibition of the EOG peak amplitudes were: n-butyl bromoacetate 41.7 ± 5.9%; n-butyl propionate 36.3 ± 11.7%; ethyl propionate 33.3 ± 11.7%; t-butyl propionate 29.3 ± 11.9%; 1,8-cineole 13.0 ± 12.4%, (mean ± S.E.M. 2 frogs).

n-Butyl bromoacetate interacted with olfactory receptors to give differential inhibition of the EOG responses with the same specificity as ethyl bromoacetate, but at a slower rate.

t-Butyl bromoacetate caused a non-specific reduction in the EOG responses. The EOG peak amplitude to t-butyl propionate was consistently reduced by a greater amount than the responses to other odorants, but the difference was too small to be regarded as significant. t-Butyl bromoacetate does not seem to act as an affinity labelling odorant.

4.3 vi) Recovery of EOG responses

During a period of four hours following treatment of the epithelium with the affinity labelling odorants described here, there was no recovery of the EOG responses. The labelling appears to be irreversible.

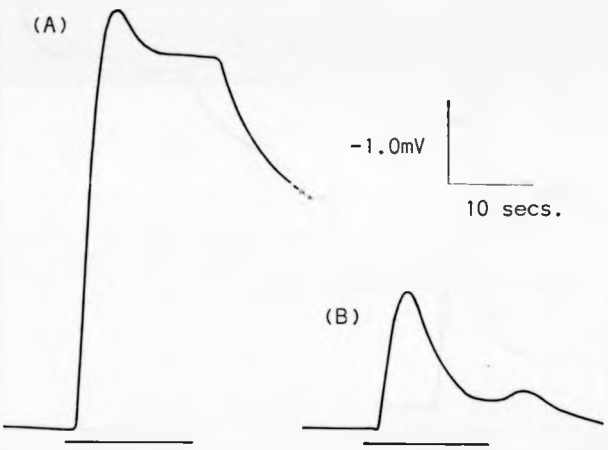
4.3 vii) Changes in EOG waveform as a result of labelling

Figures (47) and (48) show the effects of ethyl bromoacetate and t-butyl bromoacetate on the waveform of the EOG responses they elicit. In both cases the amplitude of the plateau component of the EOG is more reduced than the amplitude of the initial peak and a small, negative, 'off-response' appears. These changes were consistently found in the EOG responses to all the affinity labelling odorants tried.

The waveform of the EOG's to the other odorants used, Figure (49), were not affected in this manner, perhaps because they had higher peak/plateau ratios to start with.

FIGURE (47)

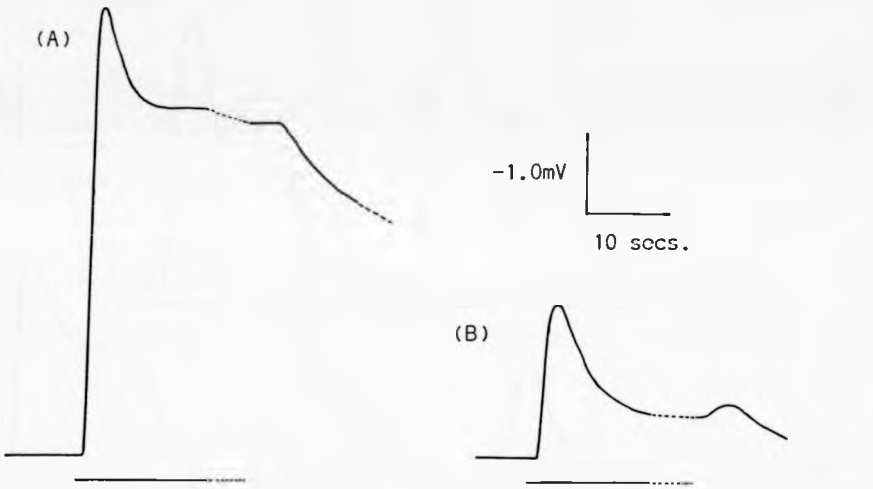
Ethyl bromoacetate



Changes in the waveform of EOG responses to ethyl bromoacetate as a result of labelling;
A) before labelling, (i.e. the first EOG in response to ethyl bromoacetate)
B) after 90 seconds labelling, (i.e. the seventh, 15 second stimulation with ethyl bromoacetate).
Horizontal bar indicates stimulus duration.

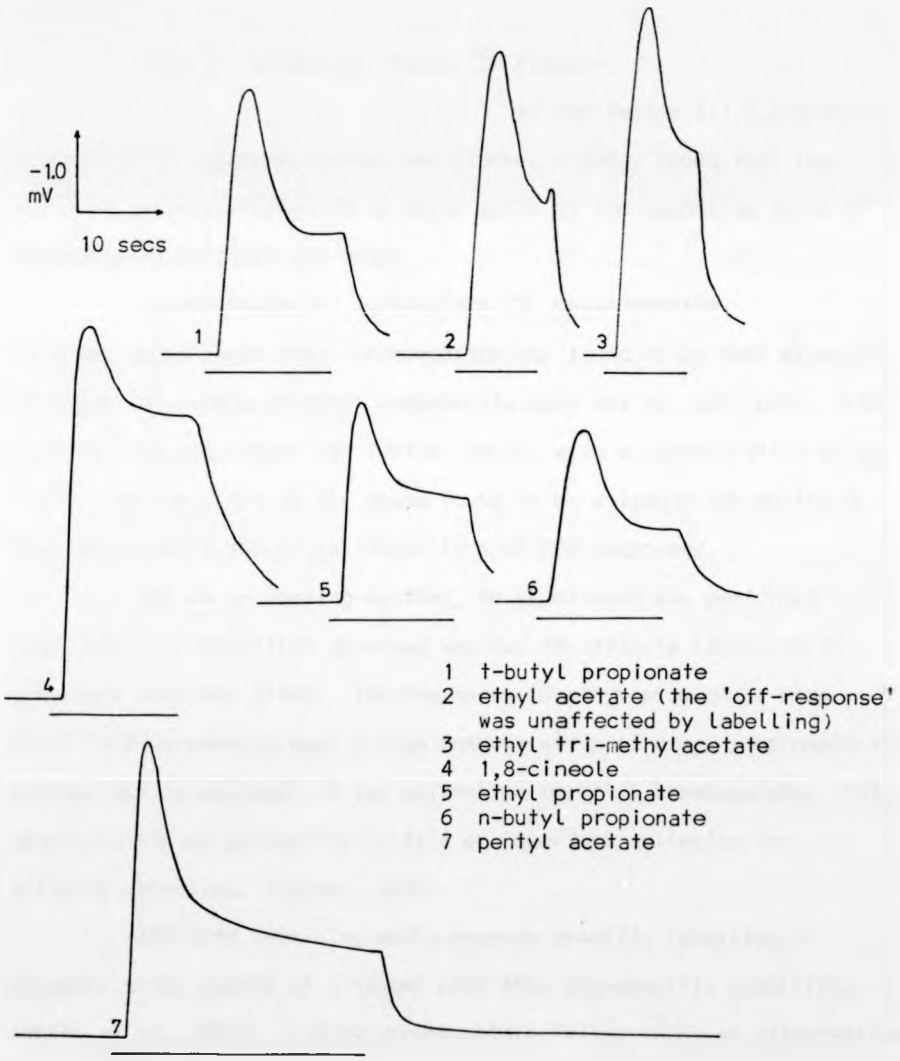
FIGURE (48)

t-butyl bromoacetate



Changes in the waveform of EOG responses to t-butyl bromoacetate as a result of labelling;
A) before labelling
B) after 900 seconds labelling
Bar indicates stimulus duration (only first 15 seconds and after stimulus switched off shown).

FIGURE (49)



EOG responses to odorants used in affinity labelling experiments. Horizontal bars indicate stimulus duration.

4.4 Discussion

The first set of experiments compared the relative abilities of ethyl chloroacetate, ethyl bromoacetate and ethyl iodoacetate to cause selective inhibition of EOG responses. According to Hendrickson et al, (1970), the reactivity of haloacetates towards nucleophiles follows the order

iodo- > bromo- > chloro- > fluoro-

in the ratios 3:1:0.2:0.0001, respectively. However, Korman and Clarke, (1956), found that the ratio of carboxymethylation of amino acids by the magnesium salts of haloacetates followed the order

bromoacetate > iodoacetate > chloroacetate.

In these experiments ethyl bromoacetate was found to be most effective, although the sample of ethyl iodoacetate used was not very pure. Ethyl bromoacetate was chosen for further study, with a concentration of ca. 1.6 microMoles/litre of air being found to be suitable for giving a high degree of differential inhibition of EOG responses.

Before proceeding further, an experiment was performed to show that the inhibition observed was due to affinity labelling of olfactory receptor sites. The responses to ethyl propionate were shown to be protected when a high concentration of ethyl propionate was present during exposure of the epithelium to ethyl bromoacetate, This demonstration of protection fulfils an important criterion for affinity labelling, (Singer, 1970).

Affinity labelling works because specific labelling of receptor sites occurs at a faster rate than non-specific labelling, (Wofsy et al, 1962). t-Butyl bromoacetate failed to cause differential

inhibition of the EOG responses. It has the same chemical specificity as ethyl bromoacetate because it has the same reactive group. It has a minty odour and will therefore bind to a different range of receptor sites to those which bind the fruity-smelling ethyl bromoacetate. That it did not differentially inhibit responses to itself may indicate that a nucleophilic amino acid residue suitably disposed for alkylation is not present in the receptor sites that recognise minty odorants. That it did not differentially inhibit the responses to ethyl propionate indicates that the effects caused by ethyl bromoacetate were indeed due to affinity labelling and not to the presence of a particularly reactive* amino acid residue in the receptor sites. n-Butyl bromoacetate, which has a similar odour quality to that of ethyl bromoacetate, caused a similar pattern of inhibition of EOG responses to that caused by ethyl bromoacetate.

The non-specific reduction in EOG responses, that occurred at a slower rate than the affinity labelling, could have been caused by non-specific labelling of olfactory receptors, enhanced mucus secretion, damage to the transduction mechanism or inhibition of metabolic processes.

Inhibition of metabolic processes would be expected to occur, (Webb, 1966). However, experiments by Getchell, (1971), have shown that inhibitors of glycolysis or oxidative phosphorylation do not immediately affect EOG production. Getchell, (1971), also found that ouabain, which inhibits Na^+, K^+ - ATPase, caused diminution of the EOG responses 'much more slowly' than her group specific reagents, experiments with

* For example, the active site of ribonuclease possesses a uniquely reactive histidine residue which enables a highly specific reaction to occur with bromoacetic acid, (Barnard and Stein, 1959).

which took about 20 minutes. The experiments reported here lasted, typically, for two hours, so inhibition of the electrogenic sodium pump may be the cause of some of the non-specific inhibition of the EOG's. Enhanced mucus secretion might also be expected to occur since the compounds used as affinity labelling odorants are lachrymators.

The effects described above could not have been responsible for the specific labelling that was observed.

One problem that can be encountered with affinity labelling is hydrolytic decomposition of the reagent. In these experiments the labelling molecules in the mucus were continuously being replaced from the vapour phase so any decomposition would have to occur in seconds, or fractions* of a second, to be of any significance at all. Barnard and Stein, (1959), found that the rate of hydrolytic decomposition of bromoacetic acid was 0.2% hour, (pH7.0, 37^{o}C). To see if ethyl bromoacetate was decomposed a sample was shaken with an excess of Frog's Ringer solution for 30 minutes. Ether extracts of the mixture, analysed by G.L.C., showed only a single peak which corresponded with ethyl bromoacetate.

The specificity of labelling by ethyl bromoacetate was examined by comparing the inhibition of the responses to a total of

* Ottoson, (1956), found the EOG latency to be about 200 milliseconds. It must take less than this for odorant molecules to reach the receptor sites.

eight odorants, one of which was chosen as a control, (1,8-cineole), the others being esters. The results, as in Table X, were:

Odorant	Odour Quality	% Inhibition of EOG Responses ± 1 S.D.
1,8-cineole	Camphoraceous	23.0 ± 8.7
t-butyl propionate	Peppermint	27.4 ± 8.0
pentyl acetate	Fruity, bananas	29.1 ± 8.5
ethyl tri-methyl acetate	Peppermint, sweet	34.8 ± 11.6
n-butyl propionate	Fruity, peardrops, bananas	36.9 ± 7.9
ethyl propionate	Fruity, apples	49.7 ± 3.9
ethyl acetate	Fruity, apples	51.1 ± 10.5
ethyl bromoacetate	Fruity, apples	72.3 ± 6.5

A close correlation was found between EOG response inhibition and molecular structure and odour quality. With the fruity smelling esters, the degree of inhibition was lower for those with longer alkyl side-chains, probably because the specificity of binding of esters to olfactory receptors may overlap less with that of ethyl bromoacetate as their structures become progressively more dissimilar to that of ethyl bromoacetate.

The odorants chosen for the specificity study were taken from those in the structure-activity relationship study in the previous chapter so that the results of the labelling experiments could be seen in context. Three odour types were examined: fruity, minty and camphoraceous. It will be interesting to see the effects of ethyl

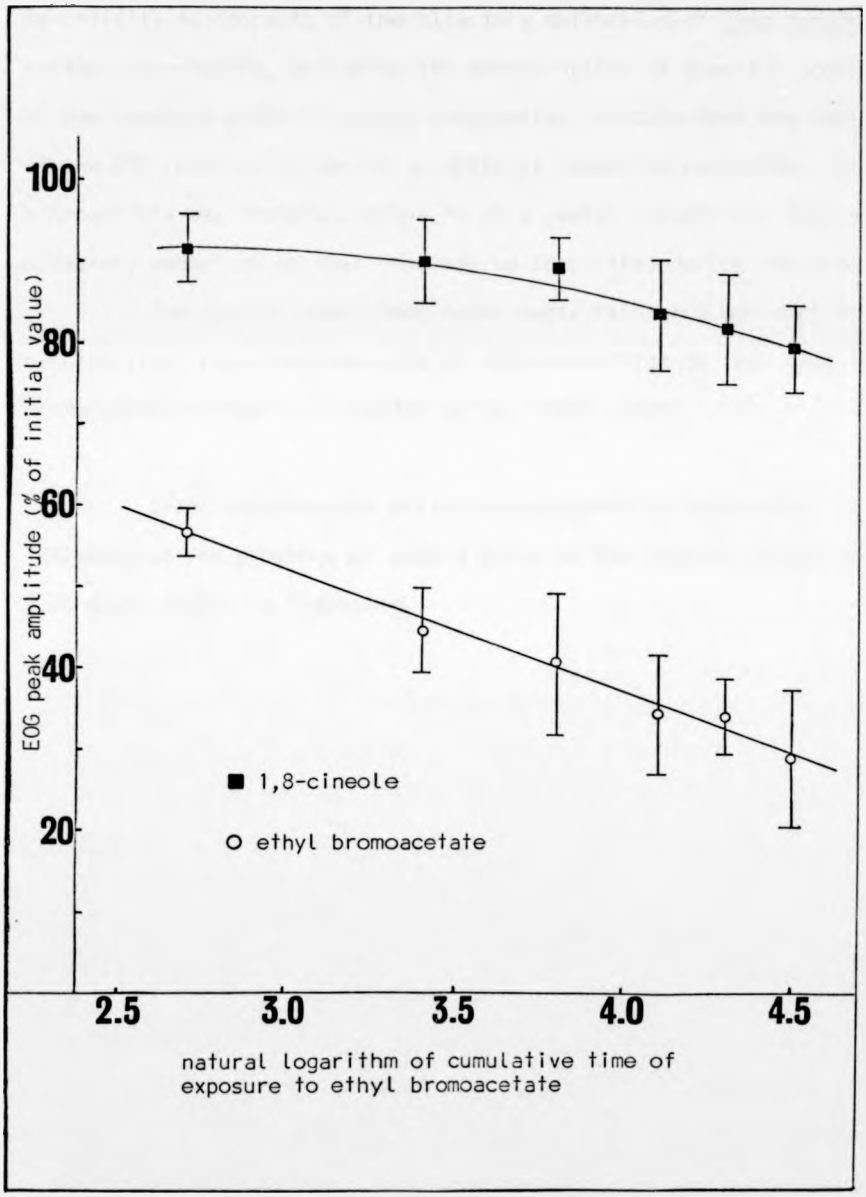
bromoacetate on the responses to a wider variety of odorants*. It would also be interesting to see its effects on single unit responses. Cross-protection experiments could be used to examine the degree of overlap of the binding sites for various odorants.

A kinetic analysis of the results, (Figure (50)), reveals that labelling by ethyl bromoacetate, as expressed by its effects on the EOG response, is linear with respect to the logarithm of the time of labelling. Plotted on the same axes, the kinetics of inhibition of the response to 1,8-cineole appears to be different, but this may be due to the parts of the two curves shown not being comparable. (It should be noted that although a curved line has been drawn through the means of the values for 1,8-cineole, the error bars would allow a straight line to be drawn).

Figure (50). Kinetics of inhibition of the EOG responses to 1,8-cineole and ethyl bromoacetate, with affinity labelling by ethyl bromoacetate. Ordinates: EOG peak amplitude remaining. Abscissa: natural logarithm of cumulative time of exposure of the epithelium to ethyl bromoacetate. The points were obtained by averaging the curves shown in Figures (37), (42), (43) and (44), and so represent the mean of 4 experiments $\pm$ 1 S.D., total of 15 frogs.

* Work along these lines is being carried out in our laboratory.

FIGURE (50)



Kinetics of inhibition of EOG responses by ethyl bromoacetate.

Ethyl bromoacetate has been shown to cause a concentration dependent, time dependent, irreversible, selective reduction in the sensitivity to odorants of the olfactory epithelium of Rana temporaria. Further experiments, including the demonstration of specific protection of the receptor sites for ethyl propionate, indicate that the inhibition of the EOG response is due to an affinity labelling mechanism. Ethyl bromoacetate may therefore prove to be a useful reagent for tagging olfactory receptors so that they may be identified during isolation.

The results from these experiments reinforce our working hypothesis of regarding odorants as regulatory ligands that bind to proteinaceous olfactory receptor sites, (Dodd, 1976).

Ethyl bromoacetate alkylates nucleophilic amino acid residues, so the presence of such a group in the receptor sites that bind small esters is indicated.

CHAPTER 5 THE ROLE OF CYCLIC NUCLEOTIDES IN THE OLFACTORY TRANSDUCTION MECHANISM

5.1. Introduction

Kurihara and Koyama, (1972), and Bitensky et al, (1972), found a high degree of adenylate cyclase activity to be present in homogenates of rabbit olfactory epithelium, (5.7 and 4.7 nanomoles cAMP/mg protein/10 mins., respectively).

Minor and Sakina, (1973), obtained EOG responses from Rana temporaria and Rana ridibunda by applying odorants in liquid phase. Similar, but slower responses were obtained to solutions of 3',5'-cyclic AMP* and its more membrane permeable dibutyryl derivative**. 2',3'-cyclic AMP and 5'-AMP did not elicit responses.

They investigated the effects of theophylline, papaverine and imidazole on the EOG's elicited by solutions of odorants. Theophylline and papaverine are cyclic nucleotide phosphodiesterase inhibitors and will therefore cause increase in intra-cellular levels of cyclic AMP. Imidazole has the opposite effect by stimulating phosphodiesterase activity.

Phosphodiesterase inhibitors were found to reduce the peak amplitude and increase the duration of EOG responses. Imidazole caused a small increase in EOG peak amplitude and reduced the duration of the response, (see Figure (51) overleaf). The effects were reversible.

* 3',5'-cyclic adenosine monophosphate

** $N^6,O^{2'}$-dibutyryl cyclic adenosine monophosphate
This compound is also less susceptible to phosphodiesterase activity.

FIGURE (51)

(a)

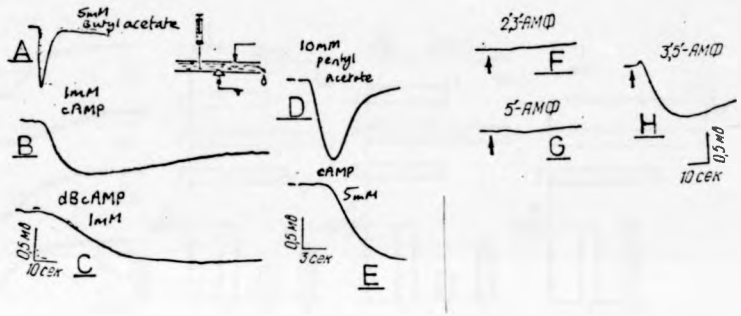


Figure (51) (a) Responses of frog olfactory epithelium to solutions of:

A 5 mM butyl acetate

B 1 mM 3',5'-cyclic AMP

C 1 mM dibutyryl 3',5'-cyclic AMP

D 10 mM pentyl acetate

E 5 mM 3',5'-cyclic AMP

F 5 mM 2',3'-cyclic AMP

G 5 mM 5'-AMP

H 5 mM 3',5'-cyclic AMP

FIGURE (51) cont.

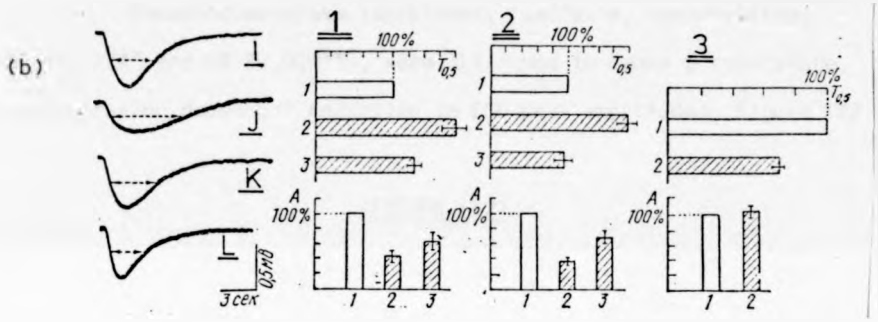


Figure (51) (b) Effects of theophylline, papaverine and imidazole solutions on the responses to odorants.

EOG's evoked by a short puff of odorous vapours in control (I), after application of 5 mM theophylline (J), after washing in Ringer's solution (K), and after application of 20 mM imidazole (L).

1 Effect of theophylline (5 mM, 10 mins) on duration (T0.5) and amplitude (A) of the EOG.

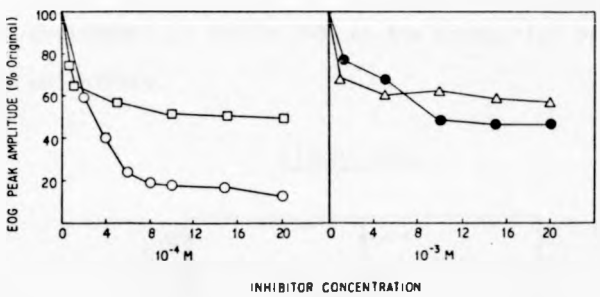
2 Effect of papaverine (1 mM, 10 mins) on the EOG.

3 Effect of imidazole (20 mM, 10 mins) on the EOG.

Menevse, (1977), and Menevse et al, (1977,b), performed a more detailed study, applying reagents in solution to the olfactory epithelium of Rana temporaria and recording EOG's in response to vapour phase pulses of odorant.

Phosphodiesterase inhibitors, (caffeine, theophylline, RO 20-1724* and SQ 20,009**), were all found to cause a reversible, concentration dependent reduction in EOG peak amplitudes, Figure (52).

FIGURE (52)



Effect of phosphodiesterase inhibitors on the amplitude of the EOG peak. The odorant was n-amyl acetate.
Δ Theophylline . ● Caffeine . ○ SQ 20009. □ RO 20-1724.

From Menevse et al, (1977,b)

* RO 20-1724 : 4 - (3-butoxy-4-methoxybenzyl)-2-imidazolidinone

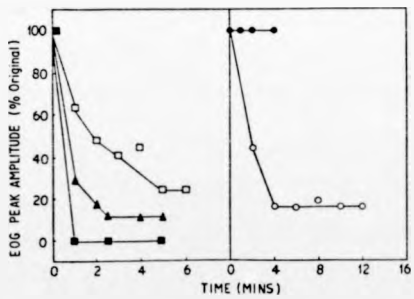
** SQ 20,009 1-ethyl-4-(isopropylidenehydrazine)-1H-pyrazole-(3,4-b)pyridine-5-carboxylic acid ethyl ester

Imidazole acted to enhance EOG responses, a 10 mM solution giving a 20% increase in peak amplitudes.

They found that cyclic AMP caused a reduction in the EOG response but the effect was much greater when membrane permeable derivatives, dibutyryl - and 8-bromo cyclic AMP were used, (Figure (53)). Dibutyryl cyclic GMP was found to cause a small decrease in the response and control compounds had no effect. A wide range of odorants was used and the effects of the various compounds on EOG responses were found to be independent of odour quality.

The results were taken as being strong evidence for the specific involvement of cyclic AMP in the production of olfactory generator potentials.

FIGURE (53)



The time course of the inhibition of the EOG peak amplitude by cAMP derivatives and phosphodiesterase inhibitors.

Symbol	Odorant	Added Compound
□	1,8-Cineole	$N^6,O^{2'}$-dibutyryl-cAMP(1mM)
▲	1,8-Cineole	8-bromo-cAMP (1 mM)
■	1,8-Cineole	$N^6,O^{2'}$-dibutyryl-cAMP (2mM)
●	n-amyl acetate	5'-AMP (1 mM)
○	ethyl n-butyrate	SQ 20009 (1 mM)

From Menevse et al, (1977,b)

The experimental work described in this chapter was performed to discover more about the mechanisms by which cyclic AMP affects the EOG responses.

In the experiments by Minor and Sakina , (1973) and Menevse et al, (1977,b), compounds causing an increase in the levels of cyclic AMP in the epithelium brought about a decrease in the sensitivity to odorants.

This could be explained if the increased cyclic AMP levels caused depolarisation of the receptor cells or, alternatively, if odorants normally act to cause a reduction in the intra-cellular cyclic AMP concentration.

There is also the possibility that cyclic AMP might not have a role in the transduction mechanism and the reduction in EOG responses may have been, for example, due to a decrease in impedance across the membrane of the supporting cells which would cause transepithelial potential differences to be reduced.

5.2. Materials and Methods

Adenosine - 3',5'-cyclic monophosphoric acid, sodium salt, (cyclic AMP); guanosine -3',5'-cyclic monophosphoric acid, sodium salt, (cyclic GMP); $N^6,O^{2'}$-dibutyryl adenosine -3',5' - cyclic monophosphoric acid, sodium salt, (dibutyryl cyclic AMP); $N^2,O^{2'}$ - dibutyryl guanosine - 3',5'-cyclic monophosphoric acid, sodium salt, (dibutyryl cyclic GMP); 8-bromo adenosine - 3',5' - cyclic monophosphoric acid, sodium salt, (8-bromo cyclic AMP); and theophylline were obtained from the Sigma Chemical Company, St. Louis, MO, U.S.A. 1-ethyl-4 (isopropylidenehydrazine)-1H-pyrazole-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester hydrochloride, (SQ 20,009), was a kind gift from the Squibb Pharmaceutical Company Ltd.

Pentyl acetate, used as an odorant and as a reagent, and inorganic salts and nutrients were obtained from B.D.H., Poole, Dorset.

All compounds used were of the highest purity available and used as received. Pentyl acetate was pure by G.L.C., (for conditions see section 2.5 (iv)).

All reagent solutions were made immediately before use, compounds being dissolved in Ringer's solutions with pH adjusted to 7.0 - 7.2 for frog tissue and 7.2 - 7.4 for mammalian tissue. Ion concentrations in Frog's Ringer solution were 114.2 mM Na^+, 1.9 mM K^+, 1.1 mM Ca^{++}, 115.3 mM Cl^-, 2.4 mM HCO_3^- and 0.64 mM $H_2PO_4^-$, in double distilled water with 10.1 mM glucose. Mammalian Ringer's solution, (Ringer Locke) contained 157.0 mM Na^+, 5.6 mM K^+, 2.2 mM Ca^{++}, 164.0 mM Cl^-, 3.0 mM HCO_3^-, and 10.1 mM glucose, in double distilled water. It was gassed with 95% O_2/5% CO_2.

Animal and tissue preparation has been described in General Materials and Methods, pages 51-55. Experiments on *in vivo* frog were carried out at room temperature, (20 ± 2°C). *In vitro* experiments were performed at 18 - 19°C, i.e. just below room temperature to ensure that the tissue did not become dried.

Solutions were carefully applied to *in vivo* frog olfactory epithelium from a 1 ml glass syringe with a 31-Gauge hypodermic needle which had the end filed smooth and bent to a suitable angle. Solutions were retained in the olfactory cavity by having the buccal aperture, (internal naris), blocked with a plug of Teflon sealing tape pushed up from inside the frog's mouth. Solutions were removed by gentle suction through a shaped pasteur pipette with a tip diameter of about 100 microns. The technique did not require removal of the electrode to allow for manipulation so the electrode position could be kept constant. Solutions added to the olfactory cavity were replaced with fresh solution at one minute intervals, usually with the total exposure time being 3 minutes. Such a procedure will be denoted as (3 x 1 mins.).

For *in vitro* preparations the method of reagent addition was to stop the flow of perfusate through the tissue cell and replace that which was already in the cell with the required solution. Removal of solutions was accomplished by restarting the flow of perfusing Ringer's solution which flushed out the cell in a matter of minutes , the flow rate of the perfusing solution being 1 - 2 mls/min and the volume of liquid to be replaced being 2.2 mls.

The experimental procedure followed was to obtain the mean of

at least three EOG's, usually to pentyl acetate and elicited at regular 2 - 3 minute intervals, to establish the baseline response, (100% value on graphs). Test solutions were then applied in the manner described above. With *in vivo* experiments solutions had to be removed from the olfactory cavity to allow EOG's to be elicited, a one minute gap being allowed from removal of the solution to the recording of the first EOG. This was not necessary with *in vitro* experiments, and, after addition of the test solution, EOG's were recorded at the same regular intervals as before.

Following treatment, *in vivo* frog epithelium was washed by three, consecutive, one-minute additions of Ringer's solution.

Results are only reported where the response returned to normal after treatment. Control runs were performed by adding Ringer's solution alone. Only preparations yielding stable responses in terms of EOG peak amplitude were used for experiments.

5.3 Results

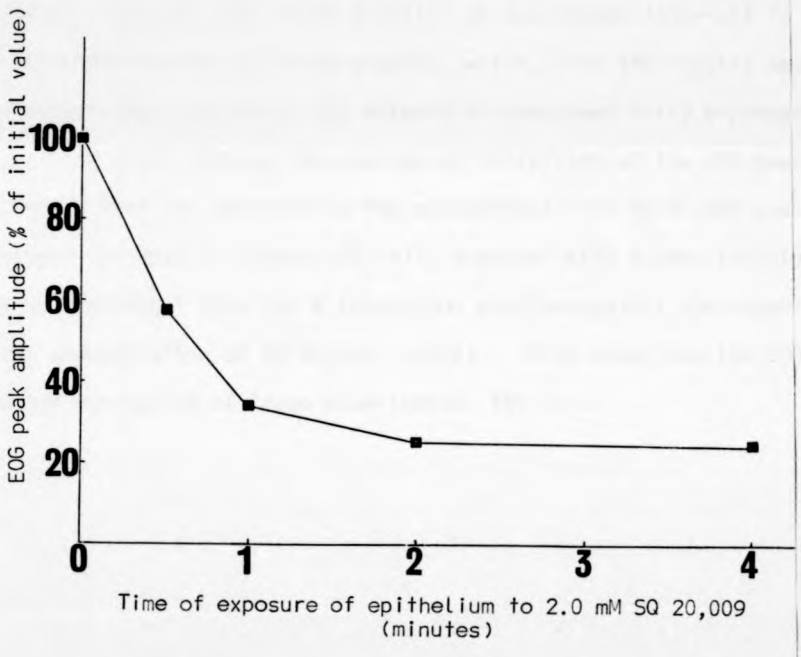
The first set of results, (5.3. (i)-(iv)), is concerned with experiments on *in vivo* frog tissue and were designed to elucidate the mechanism by which increasing cyclic AMP levels in the epithelium causes a reduction in EOG responses. SQ 20,009, a potent phosphodiesterase inhibitor, (Chasin and Harris, 1972), was chosen as the tool for manipulating cyclic AMP levels. The first experiment was to repeat the results of Menevse, (1977), and Menevse *et al*, (1977), who found that SQ 20,009 caused a 50% inhibition of the EOG peak amplitude at a concentration of 0.51 $\pm$ 0.14 mM.

5.3 i) Effects of SQ 20,009 on EOG responses

Ringer's solution was found to cause a *ca*. 7% reduction in EOG peak amplitudes to pentyl acetate. Results shown in Figures (54) and (55) are expressed relative to this.

First the time course of the appearance of effects of SQ 20,009 was determined. 2.0 mM SQ 20,009 in Ringer's solution was applied to the epithelium for 30 seconds, removed, and EOG's were recorded. Then the epithelium was washed until the peak amplitude had recovered to its 100% value. The procedure was repeated with the time of exposure to 2.0 mM SQ 20,009 being increased to 60, 120, and 240 seconds. The results are shown in Figure (54).

FIGURE (54)



Time course of inhibition of EOG peak amplitudes by 2.0 mM SQ 20,009.
Odorant: pentyl acetate.
n = 1

Inhibition of the EOG response was rapid and nearly maximal in one minute. The effects were completely reversible.

The concentration dependence of the inhibition of EOG responses was investigated by applying successively higher concentrations of SQ 20,009 to the epithelium. Solutions were added to the olfactory cavity and replaced with fresh solution at one minute intervals to give a total exposure time of three minutes, which, from the results above, should have been sufficient for effects to have been fully expressed.

A graph showing the increasing inhibition of the EOG peak amplitudes that was observed as the concentration of SQ 20,009 was increased is shown in Figure (55,(a)), together with a semi-log plot showing a straight line for % inhibition plotted against the logarithm of the concentration of SQ 20,009, (55(b)). Also shown are the EOG's recorded during one of these experiments, (55 (c)).

FIGURE (55(a))

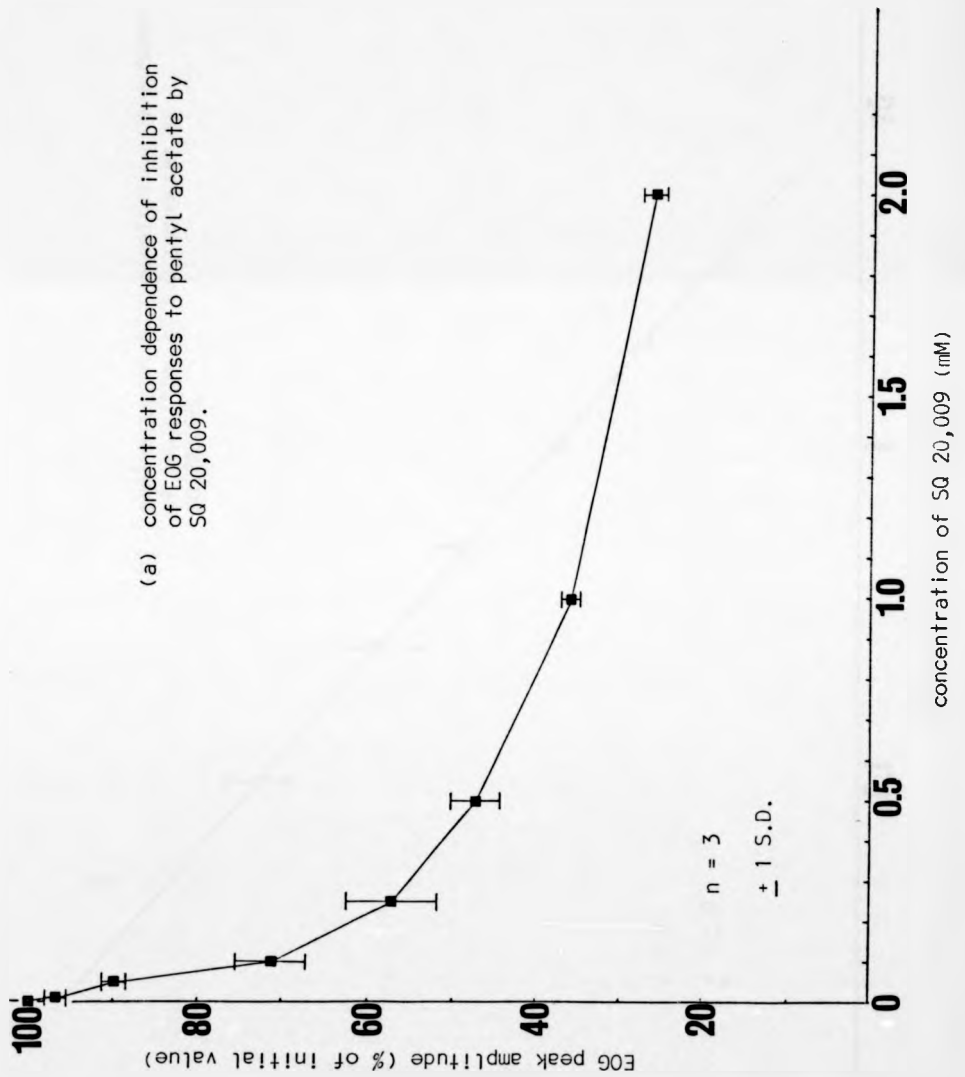
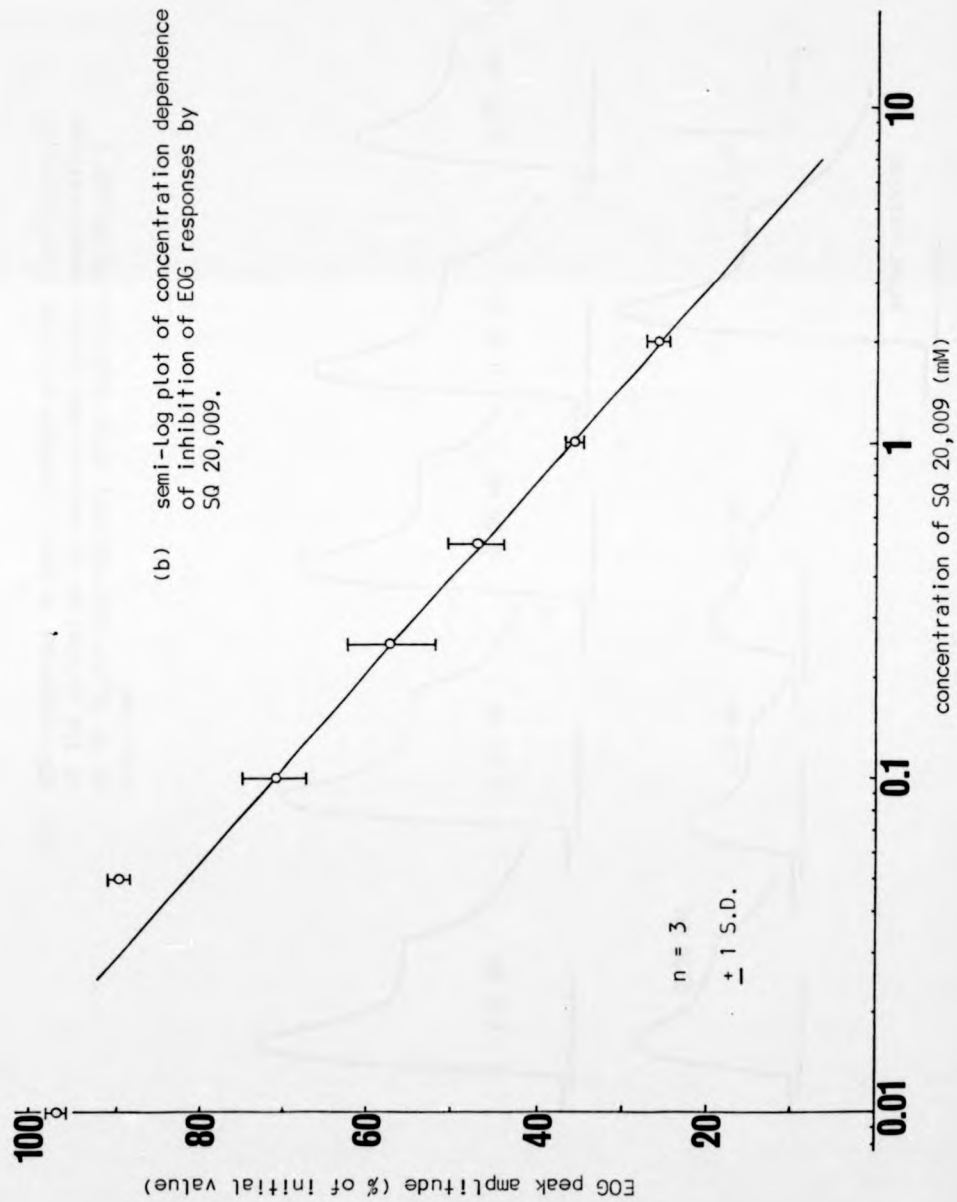

(a) concentration dependence of inhibition of EOG responses to pentyl acetate by SQ 20,009.
EOG peak amplitude (% of initial value)
100
80
60
40
20
0
0.5
1.0
1.5
2.0
concentration of SQ 20,009 (mM)
n = 3
± 1 S.D.

FIGURE (55 (b))


(b) semi-log plot of concentration dependence of inhibition of EOG responses by SQ 20,009.
EOG peak amplitude (% of initial value)
100
80
60
40
20
0.01
0.1
1
10
concentration of SQ 20,009 (mM)
n = 3
± 1 S.D.

(c) EOG responses to pentyl acetate elicited after exposure of the epithelium to successively higher concentrations of SQ 20,009 and recovery after washing with Ringer's solution.

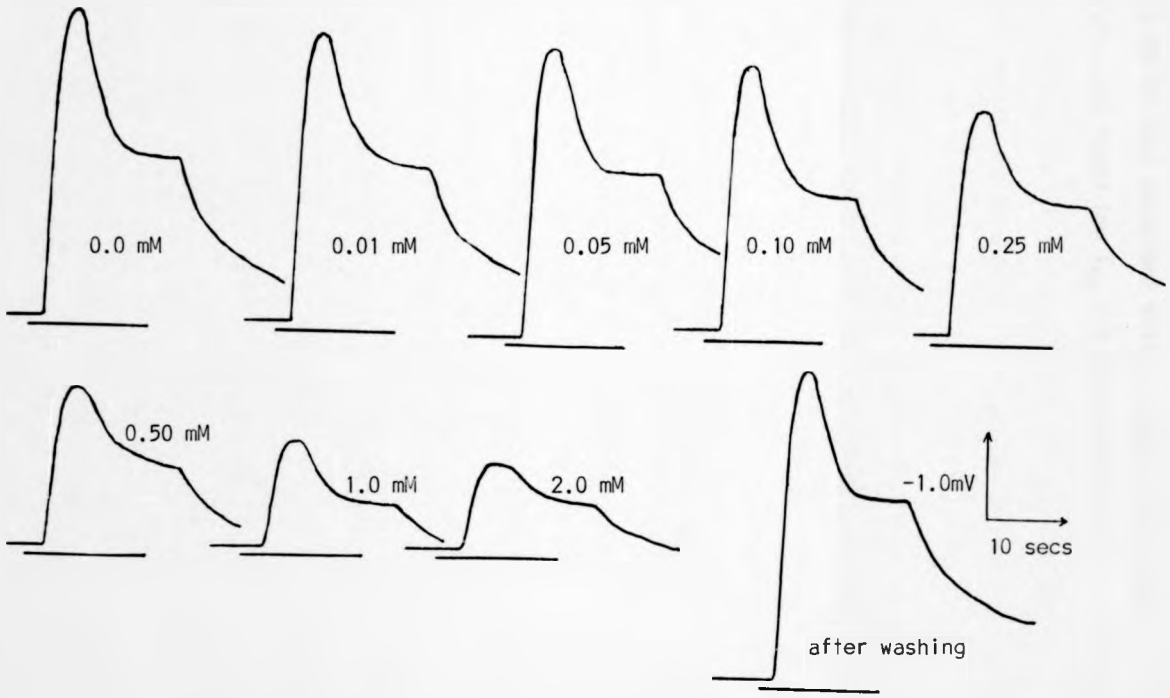


FIGURE (55 (c))

From these results the concentration of SQ 20,009 needed to cause a 50% inhibition of EOG peak amplitudes (I_{50}) was 0.40 ± 0.11 mM. This is in good agreement with Menevse, (1977), and Menevse *et al*, (1977,b), who found the I_{50} for SQ 20,009 to be 0.51 ± 0.14 mM.

5.3 ii) A comparison of the effects of SQ 20,009 on positive and negative responses from the epithelium

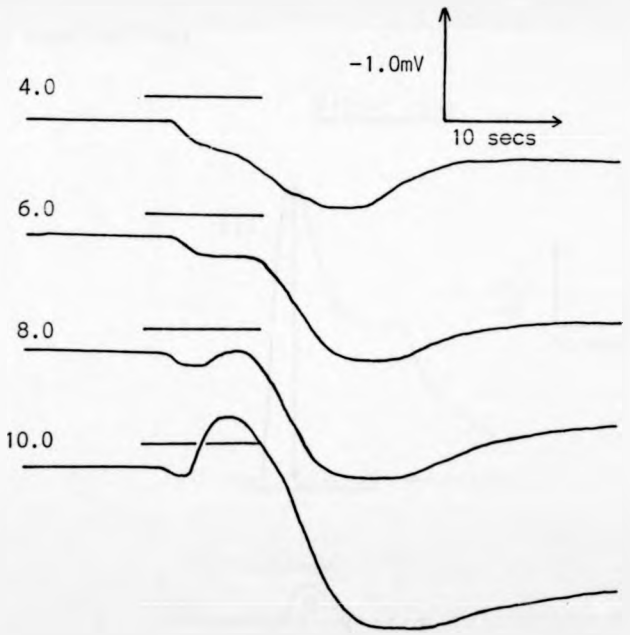
The inhibition of (negative-on) EOG responses by compounds that raise cyclic AMP levels might be explained by their causing a decrease in transepithelial impedance by increasing the permeability of the supporting cell membrane. Were this the case, positive potentials in the epithelium should be similarly affected.

A comparative study was made of the effects of 1.0 mM SQ 20,009, applied to the epithelium for 3, (3 x 1),minutes, on the negative-on EOG to pentyl acetate and the positive after-potential to ethanol.

Frog olfactory epithelium was found to give consistent responses to ethanol, similar to those reported by Gesteland, (1964). Exposure to ethanol caused inhibition of the responses to pentyl acetate. So, in these experiments, the effects of SQ 20,009 on the EOG's to pentyl acetate were determined, and only then was the frog exposed to ethanol, and the effects of SQ 20,009 on the responses to ethanol determined.

Figure (56) shows the waveform of the responses to ethanol.

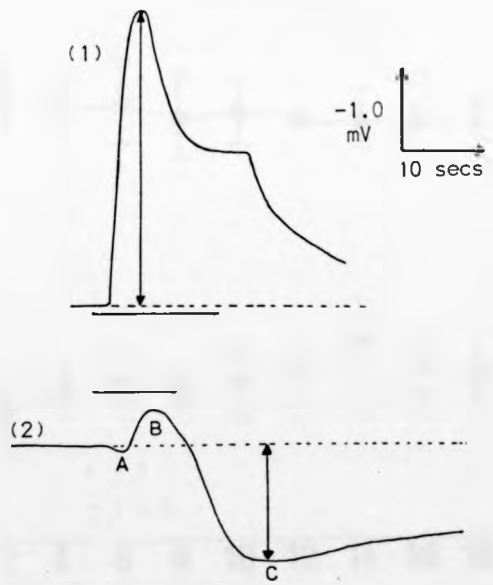
FIGURE (56)



Responses to ethanol.
Figures on left indicate relative vapour phase concentrations of ethanol
Horizontal bars indicate stimulus duration

At the lowest concentration shown, a small positive-on response was followed by a larger positive-going deflection at the cessation of stimulation. As the stimulus concentration was increased, a negative EOG appeared following the initial positive transient and the amplitude of the positive after-potential increased. The concentration as used for eliciting the last EOG in Figure (56) was used in these experiments and the amplitude of positive after-potential was monitored , (see Figure (57)), since this reached a maximum ca. 10 seconds after the cessation of stimulation, when other potentials should have declined.

FIGURE (57)

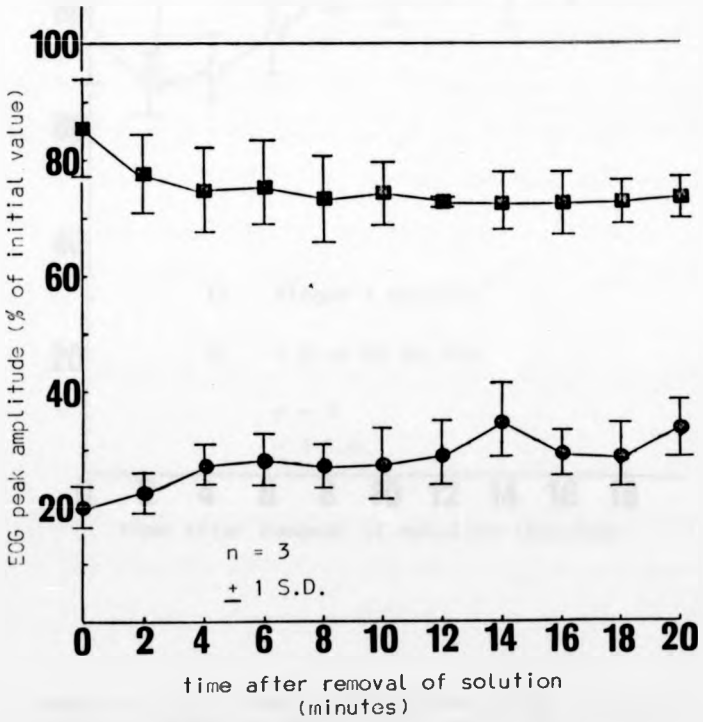


Parameters measured for positive and negative EOG's.
(1) Negative EOG to pentyl acetate, peak height measured
(2) response to ethanol. (A) initial positive transient
(B) negative phase (C) positive after potential, the amplitude of which was measured.

The average response amplitude to pentyl acetate or ethanol was determined and either a 1.0 mM SQ 20,009 solution in Ringer's, or Ringer's solution alone, as a control, were added to the olfactory cavity for 3 minutes (3 x 1 mins). The results in Figure (58) show the EOG responses after removal of the solutions, as a percentage of the responses before treatment.

FIGURE (58)

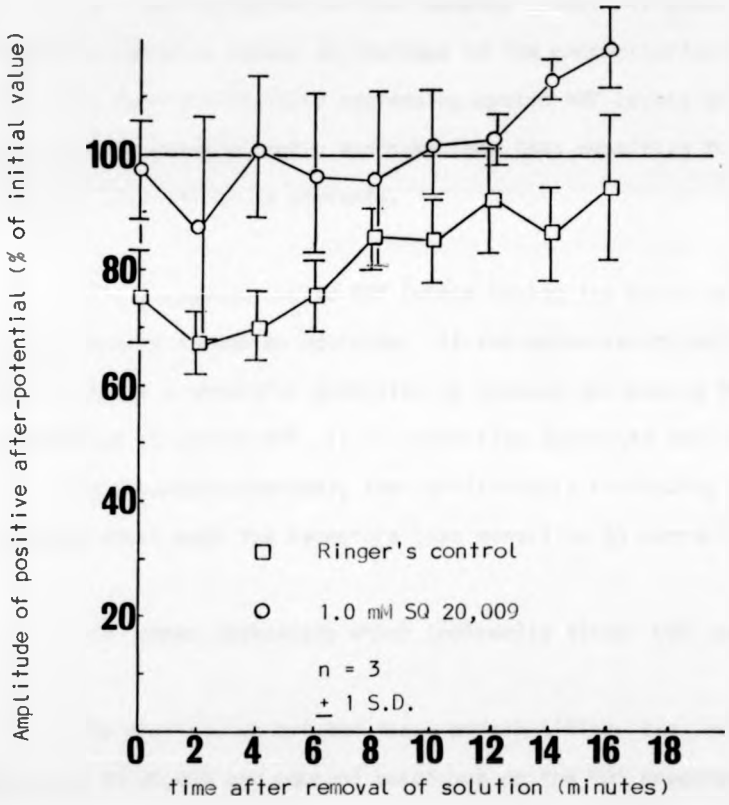
(a) Negative-on EOG



EOG responses to pentyl acetate after 3 minutes exposure of the epithelium to 1 mM SQ 20,009 (●) and Ringer's control (■).

FIGURE (58) cont.

(b) Positive after-potential



Positive after-potential to ethanol after 3 minutes exposure of the epithelium to 1 mM SQ 20,009 (O) and Ringer's control (□).

These results show that whilst SQ 20,009 inhibits the negative EOG, the positive after-potential is stimulated by ca. 20% relative to the control.

5.3 iii) Does stimulation by odorants cause an increase or a decrease in the concentration of cyclic AMP?

The inhibition of EOG responses by compounds causing an increase in cyclic AMP levels could be explained by:

a) depolarisation of the receptor cells. If odour stimulation normally causes an increase in the concentration of cyclic AMP, then artificially increasing cyclic AMP levels would depolarise the receptor cells and make them less sensitive to subsequent stimulation by odorants.

b) increased cyclic AMP levels having the opposite effect to that normally caused by odorants. If the mechanism by which odorants create a generator potential is through decreasing the concentration of cyclic AMP, (i.e. inhibiting adenylate cyclase or stimulating phosphodiesterase), then artificially increasing cyclic AMP levels would make the receptors less sensitive to odorants.

c) other mechanisms which indirectly affect EOG responses.

To distinguish between these possibilities, the individual effects of SQ 20,009 and odorant solutions on the EOG responses were determined, and then the effects of applying both simultaneously were examined to see if they acted synergistically or antagonistically.

EOG's were elicited to pentyl acetate and the effects on peak amplitude of Ringer's solutions containing the following were

determined:

i) 1.0 mM pentyl acetate

ii) 0.5 mM SQ 20,009

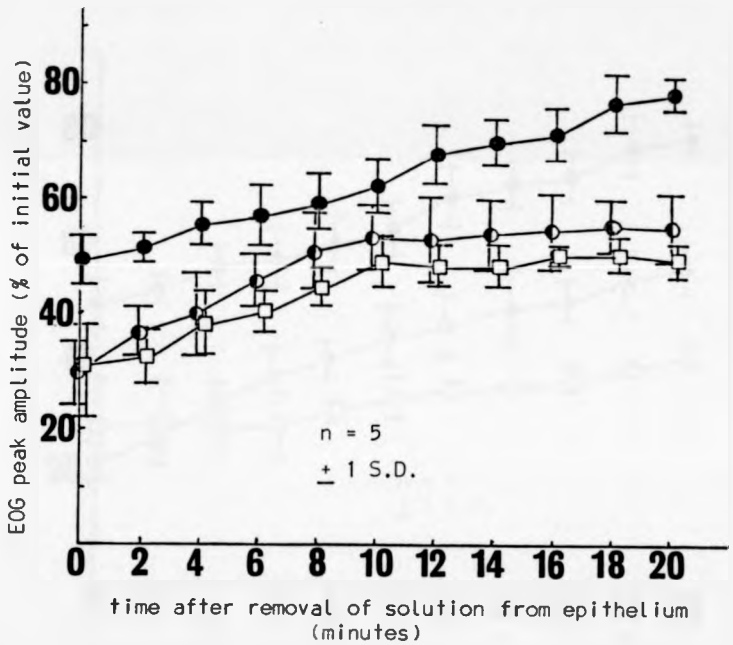
iii) 1.0 mM SQ 20,009

iv) 0.5 mM SQ 20,009 + 1.0 mM pentyl acetate

v) 1.0 mM SQ 20,009 + 1.0 mM pentyl acetate

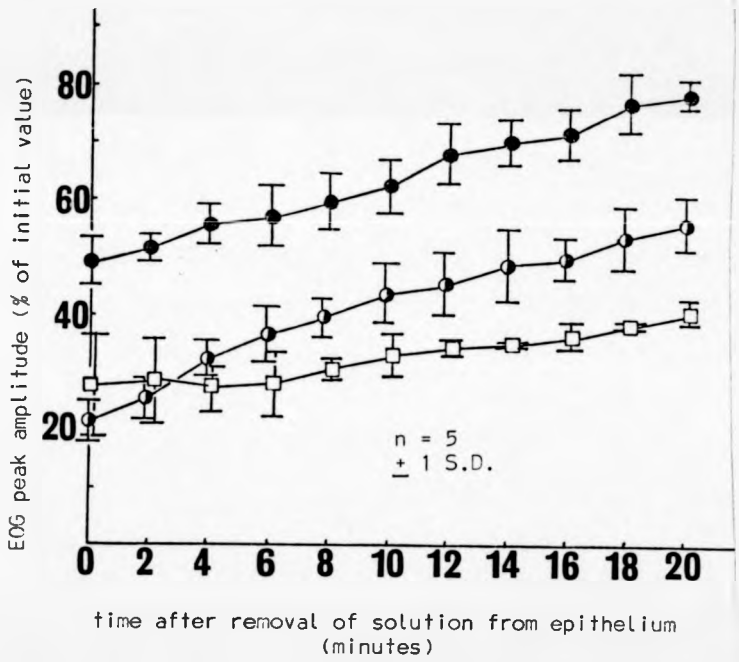
Solutions were applied to the olfactory epithelium for 3, (3 x 1), minutes and after treatment the recovery of the EOG responses was followed. All effects were fully reversible. Results are shown in Figure (59), (treatments (i), (ii), (iv)), and Figure (60), (treatments (i), (iii), (v)). All treatments were carried out on each of 5 frogs.

FIGURE (59)



Recovery of EOG responses to pentyl acetate after 3 minutes treatment with (a) (●) 1.0 mM pentyl acetate, (b) (□) 0.5 mM SQ 20,009, (c) (◐) 1.0 mM pentyl acetate + 0.5 mM SQ 20,009.

FIGURE (60)



Recovery of EOG responses to pentyl acetate after 3 minutes treatment with (a) (●) 1.0 mM pentyl acetate, (b) (□) 1.0 mM SQ 20,009, (c) (◑) 1.0 mM pentyl acetate + 1.0 mM SQ 20,009.

The EOG responses to pentyl acetate recovered at a faster rate when treated with SQ 20,009 together with pentyl acetate, (phosphodiesterase inhibitor + odorant), than when treatment was with SQ 20,009 alone.

5.3. iv) Ionic mechanisms in the presence of SQ 20,009

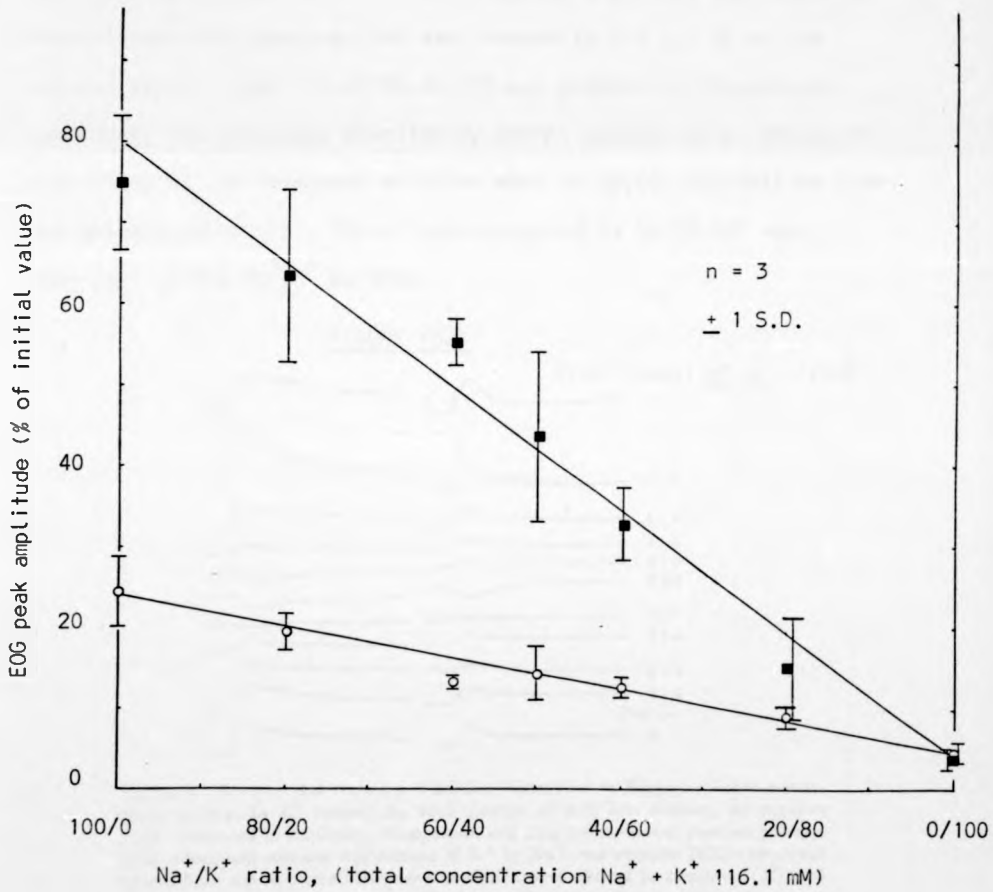
An experiment was performed to see if SQ 20,009 affects the ionic mechanisms of EOG generation. Two Ringer's solutions were made up. In one all the sodium ions were replaced by potassium ions, in the other all the potassium ions were replaced by sodium ions. Combination of the two solutions allowed Ringer's solutions to be made with the ratio of sodium ions to potassium ions varying from 100% Na^+, 0% K^+ to 0% Na^+, 100% K^+, the combined concentration of Na^+ and K^+ being a constant 116.1 mM.

Solutions containing Na^+ and K^+ ions in the ratios 100/0, 80/20, 60/40, 50/50, 40/60, 20/80 and 0/100 were applied in order to the epithelium for 3, (3 x 1), minutes. The EOG to pentyl acetate elicited two minutes after the removal of each solution was recorded. The series was then presented in reverse order to check that the effects were reversible, which in all cases they were found to be.

A second series of treatment followed, the same as the first, excepting that 1.0 mM SQ 20,009 was present in each solution.

The results from both series are plotted in Figure (61).

FIGURE (61)



Effects of varying cation ratio on inhibition of the EOG response by 1.0 mM SQ 20,009;(■) without SQ 20,009 (O) with SQ 20,009; EOG's to pentyl acetate.

The decrease in the EOG peak amplitude caused by bathing the epithelium with Ringer's solution in which Na^+ ions were progressively replaced by K^+ ions was the same as that observed by Takagi et al, (1968), see Figure (62), except at the very highest K^+ concentrations, (i.e. all Na^+ replaced by K^+), when Takagi et al, (1968), found the polarity of the response to be reversed*, whilst here it remained negative, but was reduced to 3.8 ± 1.3% of its initial value. When 1.0 mM SQ 20,009 was present in the bathing solutions, the responses elicited by pentyl acetate were reduced in proportion to the responses elicited when SQ 20,009 was omitted from the bathing solutions. The effects produced by SQ 20,009 were dependent on the Na^+/K^+ balance.

FIGURE (62)

From Takagi et al, (1968)

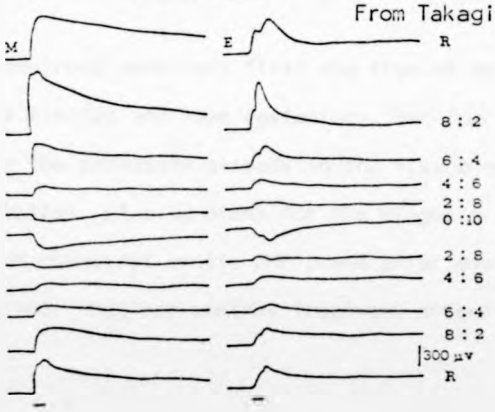


FIGURE 6. Reversal and recovery of EOG's. When Na^+ in Ringer's solution was replaced in steps by K^+ keeping the total quantity of both ions constant, the negative EOG's decreased in amplitude, disappeared, and then appeared with reversed polarity. With subsequent stepwise replacement of K^+ by Na^+, the negative EOG's recovered (shown from top to bottom). Negative-on EOG's were elicited by menthone (*M*), and negative on-off EOG's by ethyl ether (*E*). Composition of the bathing solution is given at the right as the ratios of normal Ringer's to K^+-Ringer's solution (in which Na^+ and K^+ concentrations are reversed). Thus, *R* is normal Ringer's solution and 8:2 is a mixture of eight parts Ringer's solution and two parts K^+-Ringer's solution. Short horizontal lines below columns of records indicate the time and duration (4 sec) of stimulation.

* Takagi et al, (1968), used excised dorsal olfactory epithelium which may account for the difference.

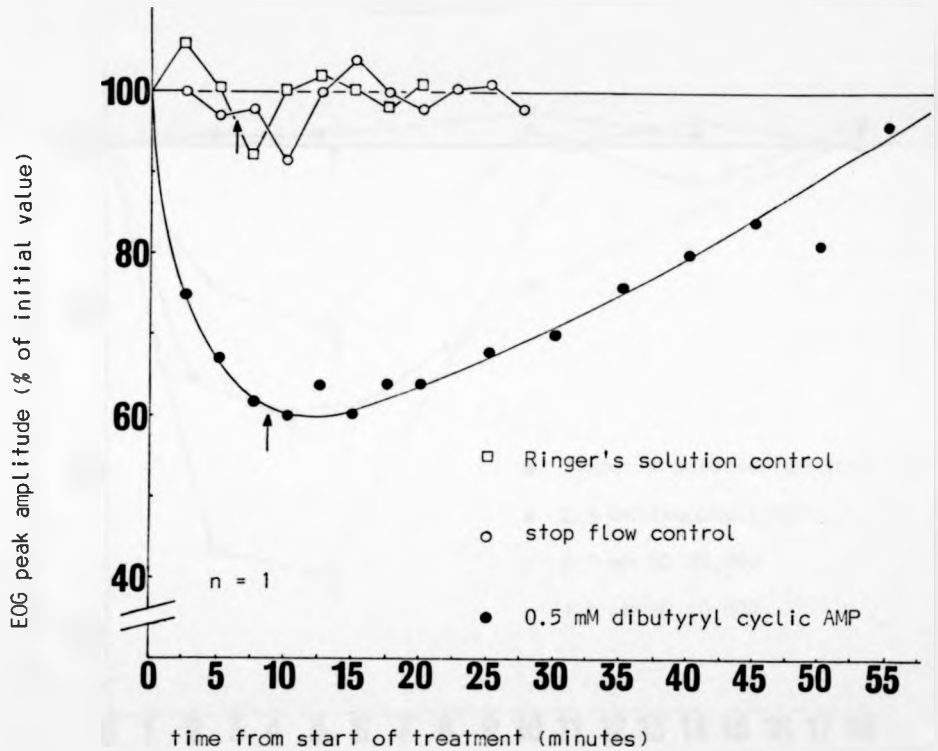
5.3 v) Effects of dibutyryl cyclic AMP, SQ 20,009 and theophylline on EOG responses from in vitro frog olfactory epithelium

Subsequent sections will deal with results obtained from experiments on in vitro sheep olfactory epithelium. So that these might be compared to results obtained from in vivo frog, the effects of two phosphodiesterase inhibitors and dibutyryl cyclic AMP on the responses from in vitro frog tissue were examined.

Figure (63) shows an experiment where in vitro frog epithelium was exposed to 0.5 mM dibutyryl cyclic AMP which caused a decline in the EOG response to pentyl acetate. The response slowly recovered when the flow of perfusate was restarted and flushed the dibutyryl cyclic AMP out of the tissue cell. (Dibutyryl cyclic AMP in Ringer's solution was added at t = 0 mins. and the flow was restarted at t = 8 mins).

Two controls were run: first the flow of perfusate was stopped for six minutes and then restarted; then the flow of perfusate was stopped and the perfusate already in the tissue cell was replaced by Ringer's solution, (i.e. a blank for the Ringer's solution which contained 0.5 mM dibutyryl cyclic AMP), and after six minutes the flow was restarted. Neither control treatment affected the response amplitude.

FIGURE (63)

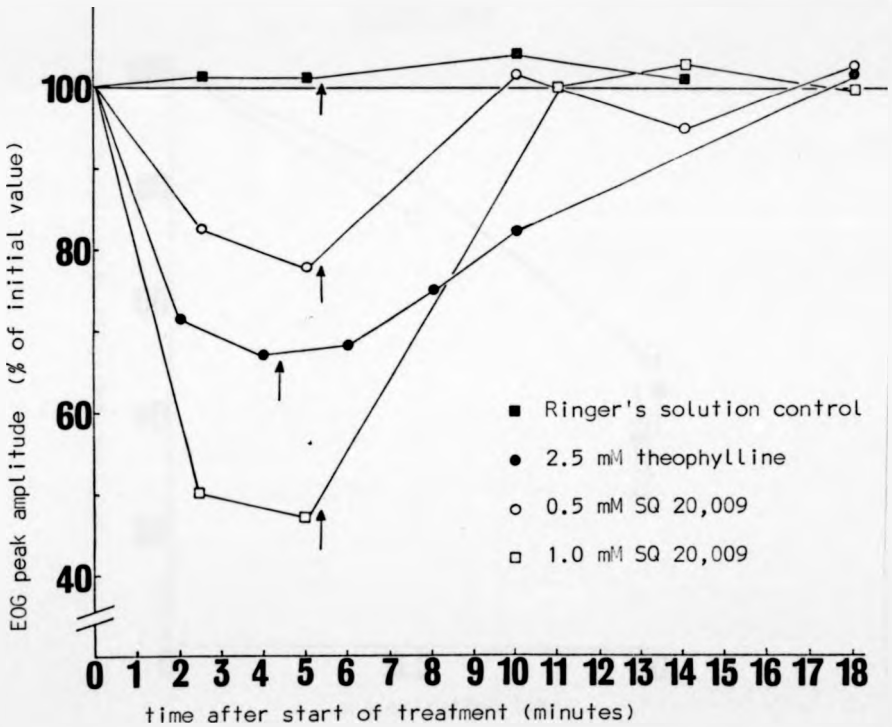


Frog olfactory epithelium *in vitro*: effects of 0.5 mM dibutyryl cyclic AMP on the EOG peak amplitude to pentyl acetate. Flow of perfusate stopped and solutions added at time = 0. Arrows indicate the point at which the flow of perfusing Ringer's solution was restarted.

Dibutyryl cyclic AMP had the same effect on *in vitro* frog olfactory epithelium as Menevse, (1977), and Menevse *et al*, (1977), found with frog olfactory epithelium *in vivo*.

The effects of Ringer's solutions containing 0.5.mM and 1.0 mM SQ 20,009 and 2.5 mM theophylline were examined, (Figure (64)), using the same procedure as for dibutyryl cyclic AMP. (Although shown in the same figure as SQ 20,009, the experiment with theophylline was carried out on tissue from a different frog and on a separate occasion).

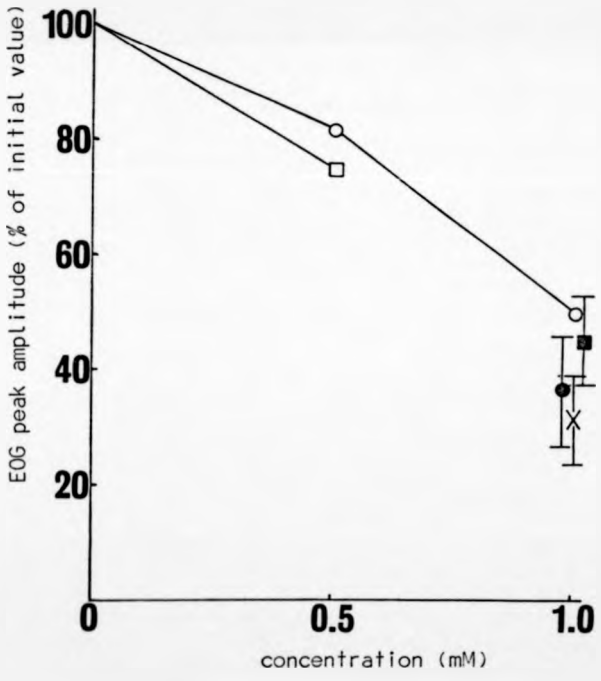
FIGURE (64)



In vitro frog olfactory epithelium: effects of phosphodiesterase inhibitors 0.5 mM and 1.0 mM SQ 20,009 and 2.5 mM theophylline, on peak amplitudes of EOG's to pentyl acetate. Flow of perfusate stopped and solutions added at time = 0. Arrows indicate the time at which the flow of perfusing Ringer's solution was restarted.

Figure (65) shows the effects of SQ 20,009 and permeable cyclic AMP derivatives on the EOG responses elicited from in vivo frog olfactory epithelium, (results taken from Menevse, 1977), compared to their effects found here on the responses from the same tissue maintained in vitro. Both sets of results relate to the EOG peak amplitudes elicited after 2.5 minutes exposure of the epithelium to the compounds.

FIGURE (65)



A comparison between in vivo and in vitro preparations of frog olfactory epithelium of the effects of 2.5 minutes exposure to SQ 20,009 or cyclic AMP derivatives on the EOG to pentyl acetate.
Frog in vitro (O) SQ 20,009, (□) dibutyryl cyclic AMP
Frog in vivo, from Menevse (1977), means + S.E.M.
(●) SQ 20,009, (■) dibutyryl cyclic AMP, (X) 8-bromo cyclic AMP.

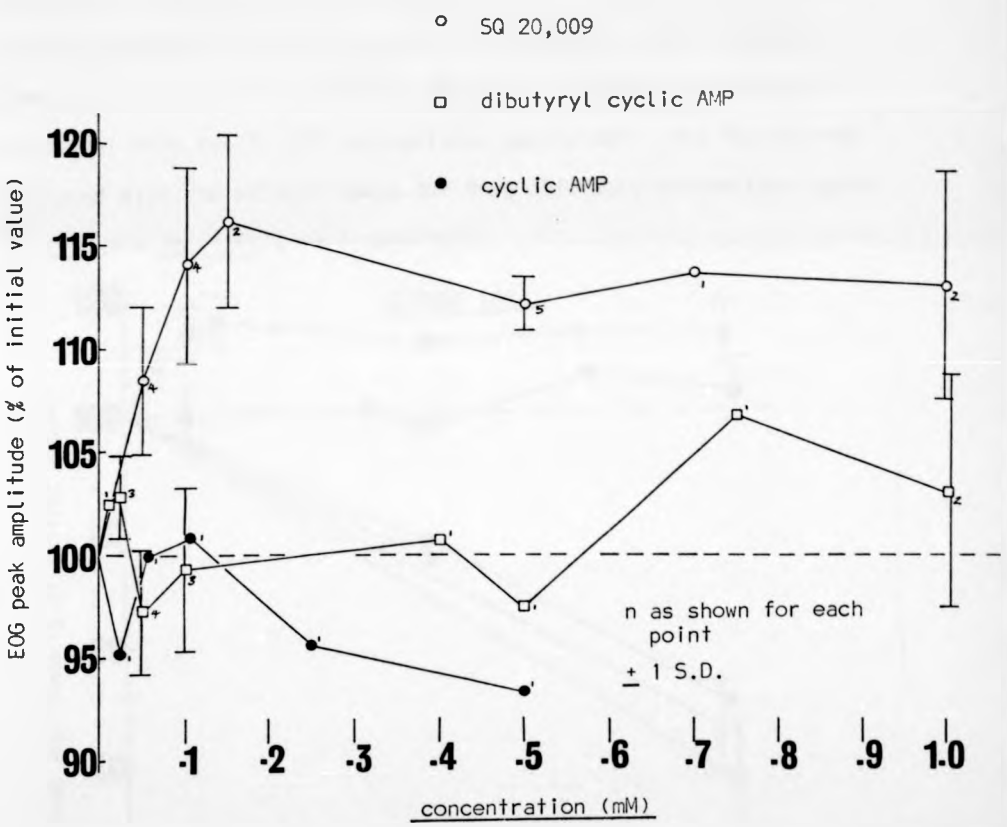
The effects of the compounds *in vivo* and *in vitro* were similar. Theophylline was found, in both preparations, to need to be at higher concentrations than SQ 20,009 to give the same degree of inhibition of the EOG responses.

5.3. vi) The role of cyclic nucleotides in the generation of EOG's in mammalian olfactory epithelium

For these experiments to be valid it was necessary to establish that the responses elicited by stimulation with odorants from in vitro mammalian olfactory epithelium were indeed EOG's. To avoid digression, the findings to support this assumption and other information gained by recording from in vitro mammalian olfactory epithelium will be reported separately in Appendix III.

Figure (66) shows the concentration dependence of the effects of SQ 20,009, dibutyryl cyclic AMP, and cyclic AMP on the peak amplitude of EOG's elicited from in vitro sheep olfactory epithelium. EOG's to pentyl acetate were recorded at regular 2.5. minute intervals. Solutions were applied by stopping the flow of perfusate and replacing that already in the tissue cell with the required solution. Points in Figure (66) show the EOG peak amplitude 2.5 minutes after addition of the solutions, expressed as a percentage of the mean peak height of 3 responses recorded immediately prior to treatment.

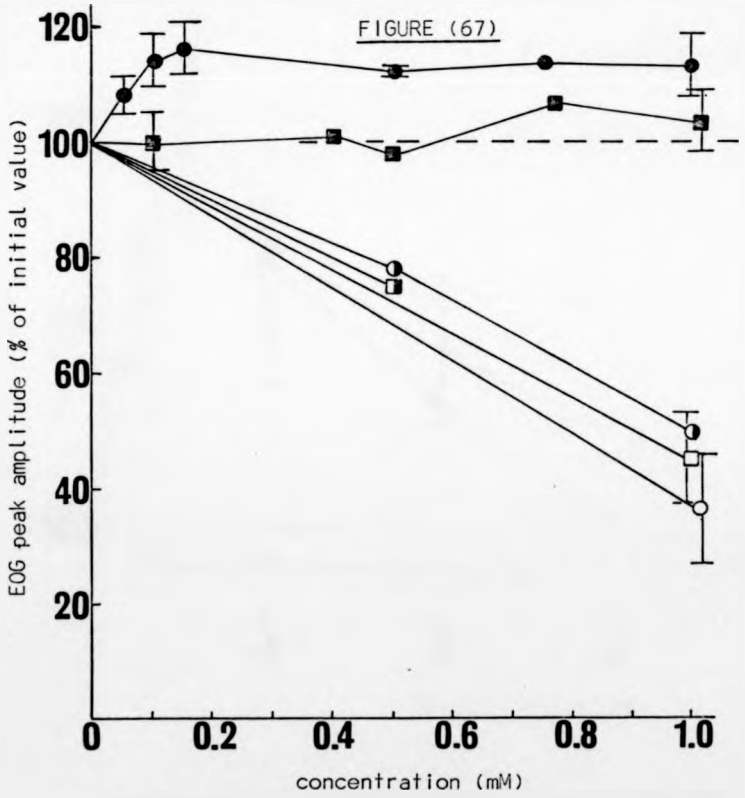
FIGURE (66)



Concentration dependence of the effects of 2.5 minutes exposure of sheep epithelium in vitro to SQ 20,009, dibutyryl cyclic AMP and cyclic AMP on EOG's to pentyl acetate.

Control experiments where the flow of perfusate was stopped, or Ringer's solution was added as a blank, after 2.5 minutes gave responses ranging from 93.3% to 101.7% of the initial value.

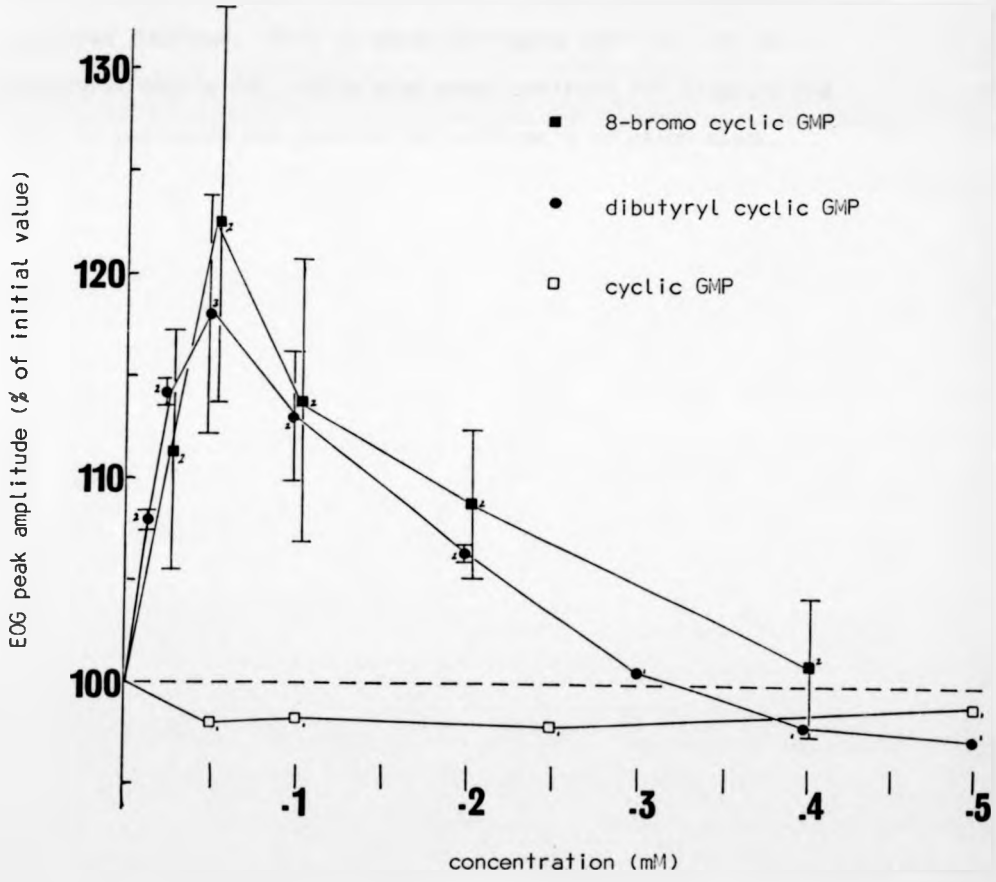
SQ 20,009 was found to cause a small increase in the EOG response, with maximum stimulation occuring at 0.15 millimolar when the EOG response was 116.2 ± 4.2% of the response before treatment, (mean ± 1 S.D. n = 2). The EOG responses remained unaffected by treatment with cyclic AMP or dibutyryl cyclic AMP. The results are compared with the effects found for frog olfactory epithelium, (both *in vivo* and *in vitro*), in Figure (67).



Peak amplitudes of EOG's to pentyl acetate from various preparations of olfactory epithelium (frog *in vivo*, frog *in vitro* and sheep *in vitro*), after 2.5 minutes exposure of the epithelium to SQ 20,009 or bibutyryl cyclic AMP. Frog *in vivo* (O) SQ 20,009, (□) dibutyryl cyclic AMP (± 1.S.D.) from Menevse, (1977). Sheep *in vitro*(●) SQ 20,009, (■) dibutyryl cyclic AMP, (± 1 S.D.) Frog *in vitro* (◑) SQ 20,009, (◨) dibutyryl cyclic AMP .

The effects of cyclic GMP and its dibutyryl and bromo derivatives are shown in Figure(68). The experimental procedure was the same as for the experiment reported in Figure(66).

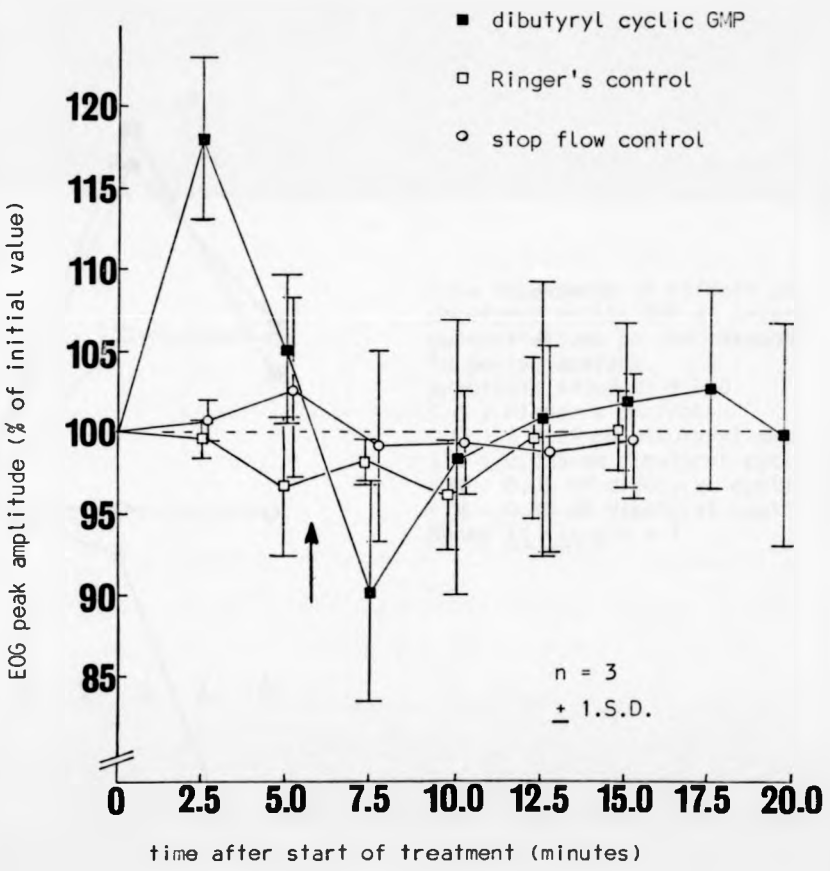
FIGURE (68)



Concentration dependence of effects of cyclic GMP and derivatives on the EOG's to pentyl acetate from sheep epithelium in vitro, which was exposed to each solution for 2.5 minutes. n as given for each point ± 1 S.D.

Both dibutyryl cyclic GMP and 8-bromo cyclic GMP stimulated the EOG response at low concentrations, (maximum at 0.05 mM). The EOG response to pentyl acetate increased to 118.0 $\pm$ 4.9% of its pre-treatment value when dibutyryl cyclic GMP was added to the tissue cell, and to 122.4 $\pm$ 8.8% in response to 8-bromo cyclic GMP. Underivatised cyclic GMP was ineffective. The stimulation of the response was time dependent, and after the initial increase the response declined. This is shown in Figure (69) for 0.05 mM dibutyryl cyclic GMP, which also shows controls for stopping the flow of perfusate and addition of a Ringer's solution blank.

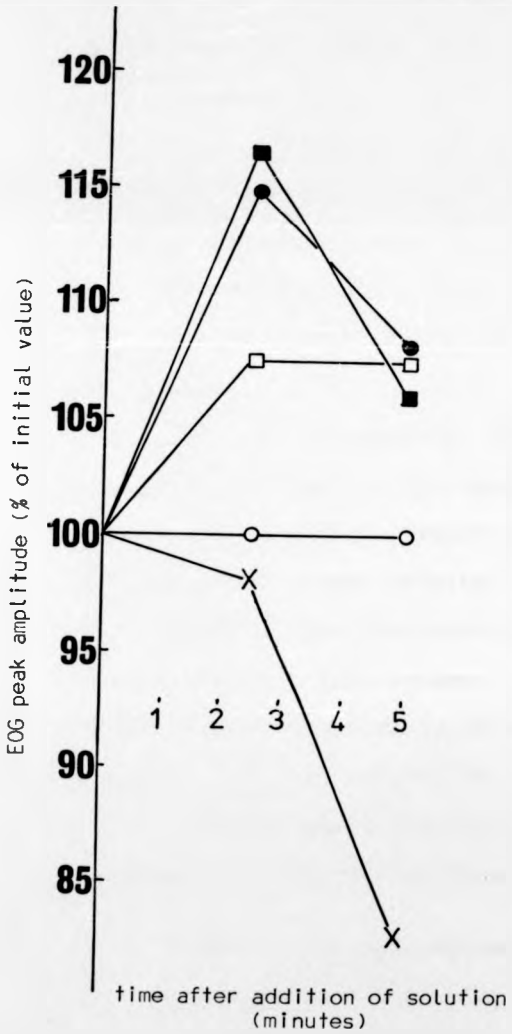
FIGURE (69)



Time dependence of effects of 0.05 mM dibutyryl cyclic GMP on EOG's to pentyl acetate from in vitro sheep olfactory epithelium.
Arrow indicates point at which the flow of perfusate was restarted.

Figure (70) shows the time dependence of the effects of dibutyryl cyclic GMP at several concentrations.

FIGURE (70)



Time dependence of effects of dibutyryl cyclic GMP at several concentrations on the responses to pentyl acetate.
Solutions added at t = 0
(O) Ringer's control
(□) 0.0125 mM dibutyryl cyclic GMP
(●) 0.025 mM dibutyryl cyclic GMP
(■) 0.05 mM dibutyryl cyclic GMP
(X) 0.40 mM dibutyryl cyclic GMP
Sheep *in vitro* n = 1

5.4 Discussion

The first experiment repeated the work of Menevse, (1977), and Menevse *et al*, (1977), who used SQ 20,009 to inhibit the EOG response. The I_{50} determined here, 0.40 ± 0.11 mM, is in good agreement with their figure of 0.51 ± 0.14 mM. SQ 20,009 is a potent phosphodiesterase inhibitor with an I_{50} of 0.002 mM for the cyclic nucleotide phosphodiesterase from rat or rabbit brain, (Chasin and Harris, 1972). The high concentration required for inhibiting the EOG response may be due to SQ 20,009 not being specific for phosphodiesterase(s) present in the olfactory epithelium. The short time taken for SQ 20,009 to give maximum inhibition of the EOG response, (see Figures (53) and (54)), indicates that diffusion is not a problem.

The second experiment compared the effects of 1.0 mM SQ20,009 on the negative EOG responses to pentyl acetate and the positive after-potentials resulting from stimulation with ethanol. The negative EOG's were inhibited by SQ 20,009, whilst the positive after-potentials were stimulated by *ca*. 20% compared to the Ringer's solution control. This increase in magnitude of the positive after-potentials implies that SQ 20,009 does not affect the negative EOG by causing a general reduction in the transepithelial impedance.

Two explanations could be put forward to account for the increase in the amplitude of the positive after-potentials.

(a) If the amplitude measured was a summation of negative and positive components, inhibition of the negative component would have made the overall response more positive.

(b) Okano and Takagi, (1974), have shown the positive after-potential to be related to Cl^- influx and "powerful secretory activity" by the supporting cells. SQ 20,009 may have enhanced this secretory activity and increased the permeability of the supporting cell membrane to chloride ions.

Possibility (a) is in accordance with the hypothesis that cyclic AMP is involved in the olfactory transduction mechanism. However, possibility (b) suggests another mechanism to account for the inhibition of EOG responses by compounds which raise cyclic AMP levels in the epithelium.

Organic solvents, which elicit positive after-potentials, have been shown to inhibit EOG responses. For example, Ottoson, (1956), found that exposure of the olfactory epithelium of Rana temporaria to a few cc's of ether or chloroform vapour blocked the EOG response to butanol when this was applied immediately afterwards. The EOG response slowly recovered and regained its initial amplitude within an hour. The effect was dose-dependent.

FIGURE (71)

From Ottoson, (1956)

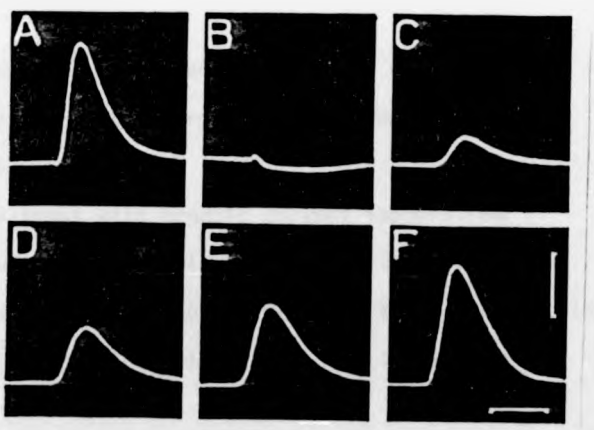


Fig. 8. The effect of ether. Records of responses to stimulation with butanol: A, before; B, immediately after ether vapour was blown into the nasal cavity; C, 10 min; D, 20 min; E, 30 min; F, 60 min later. Volume of air 1 cc. Vertical line to F 1 mV. Time-bar 2 sec.

Here, 90 seconds exposure of the epithelium to ethanol at the concentration used for eliciting positive after-potentials was sufficient to inhibit the responses to pentyl acetate by 50%. Figure (72) shows that the responses to ethanol were similar to those obtained in response to chloroform by Takagi et al, (1968).

FIGURE (72)

(a) Response to ethanol of olfactory epithelium of Rana temporaria, (this study).

(b) Response to chloroform of olfactory epithelium of Rana catesbiana, (Takagi et al, (1968)).

Okano and Takagi, (1974), showed that the secretory activity in the supporting cells in response to chloroform vapours was linked to Cl^- influx.

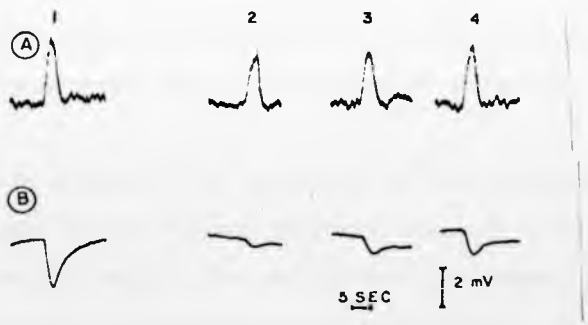
If after treatment with organic solvent vapours the supporting cells become more permeable to chloride ions, influx of sodium ions into the olfactory neurons in response to odorants would change potential gradients in the epithelium so as to cause a

flow of chloride ions into the supporting cells. The movement of Cl^- ions would tend to cancel out the charge transfer due to the influx of Na^+ ions, so that an electrode observing overall potential changes in the mucus would observe a reduced EOG. The generator potential in the neurons would be unaffected, but the EOG response would be inhibited.

This mechanism would predict that the nerve impulses, which are dependent on the generator potential, would be unaffected whilst the amplitude of the EOG would be reduced. The experiment to test this has already been performed.

Mozell,(1962), exposed the olfactory epithelium of Rana catesbiana to concentrated acetone vapour and examined the effects of this treatment on the EOG and summated neural discharge in response to geraniol. His results are shown in Figure (73).

FIGURE (73)



Effects of concentrated acetone vapour on simultaneously recorded summated neural discharge and the EOG.
(A) summated neural discharge, (B) EOG
1) responses to geraniol before acetone treatment;
2) responses to geraniol 3 mins after 4 cc of air which was passed over concentrated acetone was puffed on to the epithelium; 3) after 10 mins; 4) after 20 mins.

The EOG's to geraniol were inhibited after exposure of the epithelium to acetone, but little effect was observed on the summated discharge in the olfactory nerve.

To examine whether or not compounds which increase cyclic AMP levels inhibit EOG responses by such a mechanism, two experiments are suggested:

i) to examine the effects of phosphodiesterase inhibitors and exogenous cyclic AMP on the summated neural discharge. This may give a more direct measure of their effects on the generator potential than the EOG does.

ii) to see if increasing the concentration of cyclic AMP in the epithelium stimulates secretory activity in the supporting cells.

Dr. B.P.M. Menco of the University of Utrecht, Netherlands, is examining the second possibility using electron microscopy.

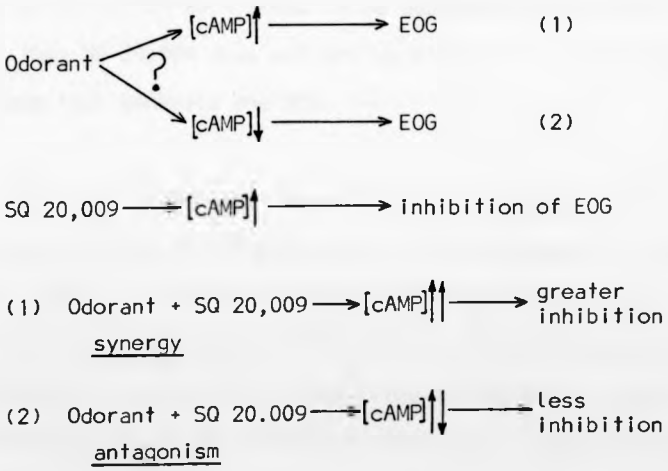
The third experiment was designed to determine whether the binding of odorants to the olfactory receptors causes an increase or a decrease in cyclic AMP levels. The inhibition of EOG responses by SQ 20,009, which raises the concentration of cyclic AMP by inhibiting phosphodiesterase activity could be accounted for in terms of either possibility.

If odorants cause an increase in the concentration of cyclic

AMP, then phosphodiesterase inhibitors and exogenous cyclic AMP would depolarise the olfactory neurons and make them less sensitive to subsequent stimulation by odorants.

Alternatively, if odorants normally act by causing a decrease in the concentration of cyclic AMP, (e.g. the decrease in the concentration of cyclic AMP caused by the binding of insulin to hepatocytes, Exton et al, 1972), then treatments which raise the levels of cyclic AMP would make the olfactory neurons less sensitive to stimulation by odorants.

The first possibility would predict a synergistic interaction between SQ 20,009 and odorant in solution, whilst the second would predict an antagonistic interaction. This is shown schematically:



where [cAMP] is the concentration of cyclic AMP
vertical arrows indicate increase or decrease in this.

When an odorant in solution, (pentyl acetate), was applied to the epithelium at the same time as SQ 20,009, the EOG response recovered more quickly. The effect of the odorant was antagonistic to that of SQ 20,009 which, assuming that cyclic AMP is involved in the transduction mechanism, would imply that odorants normally act through decreasing cyclic AMP levels in the olfactory neurons. However, were the inhibition of the EOG's to be associated with secretory activity of the supporting cells, the action of pentyl acetate in reducing the effects of SQ 20,009 could be explained by membrane stabilisation, (Seeman, 1972), inhibiting secretion.

The next experiment was performed to see if SQ 20,009 acted to perturb the ionic mechanism of EOG generation. The changes in the EOG caused by SQ 20,009 were found to be dependent on the Na^+/K^+ ratio, indicating that SQ 20,009 does not act by directly altering the pattern of ion fluxes that generate the EOG.

The final experiment investigated the effects of SQ 20,009 and cyclic nucleotides on the EOG's elicited from mammalian olfactory epithelium. Sheep tissue maintained *in vitro* was used. The olfactory epithelium from frog, maintained in the same manner, was found to respond to SQ 20,009, theophylline and dibutyryl cyclic AMP in the same way *in vitro* as was previously found when experiments were performed *in vivo*.

With sheep tissue *in vitro*, cyclic AMP and cyclic GMP, which have low membrane permeabilities, were found to have little or no effect on the EOG responses to pentyl acetate. Neither was any effect

found with dibutyryl cyclic AMP.

SQ 20,009, dibutyryl cyclic GMP and 8-bromo cyclic GMP all caused small, ca. 18% increases in the EOG responses. The initial stimulation of the EOG by these compounds was followed by inhibition. The effects were reversible.

That dibutyryl cyclic AMP did not affect the EOG response from sheep olfactory epithelium might indicate that cyclic AMP does not have a function in the olfactory transduction mechanism in this tissue. It may be that dibutyryl cyclic AMP was unable to diffuse to the receptive membrane in sheep tissue, although no such problem was encountered with frog tissue in vitro, and SQ 20,009 and cyclic GMP derivatives were effective in sheep tissue. Alternatively, the ineffectiveness of dibutyryl cyclic AMP in sheep olfactory epithelium may be because the supporting cells in mammalian olfactory epithelium do not have the secretory function that they have in amphibian olfactory epithelium and so dibutyryl cyclic AMP was unable to alter Cl^- permeability through stimulating such secretory activity.

That dibutyryl cyclic GMP , 8-bromo cyclic GMP and SQ 20,009 all stimulated the responses from sheep tissue may be significant, but since the presence of guanylate cyclase activity in the olfactory epithelium has yet to be demonstrated, any possibility that cyclic GMP is involved in the olfactory transduction mechanism would be highly speculative. These results indicate that it may be worthwhile to look for guanylate cyclase activity in olfactory tissue.

Adenylate cyclase activity has been found in mammalian olfactory epithelium: in rabbit tissue, (Kurihara and Koyama, 1972 ,

and Bitensky et al, 1972); and in bovine tissue, (Menevse, 1973). These studies did not determine in which cell type in the epithelium the adenylate cyclase activity was located, nor was modification of the activity by odorants demonstrable, although this may have been due to disruption of the enzyme. The experiments reported here would indicate, if cyclic AMP is involved in the olfactory transduction mechanism in the frog, that odorants act by causing a decrease in its concentration.

The possibility that cyclic AMP is involved in controlling the secretory activity of the supporting cells, which has in turn been linked to Cl^- fluxes, (Okano and Takagi, 1974), provides an alternative explanation for the modification of the EOG response by compounds which alter cyclic AMP levels. This hypothesis can be put to the test by examining the effects of altering the concentration of cyclic AMP on the simultaneously monitored EOG and summated neural discharge in response to odorants.

6. Conclusions

A structure activity relationship study of simple alkyl esters has been used to identify the structural features responsible for the fruity odour quality normally associated with esters. Observations indicate that to have a fruity odour these compounds require an exposed, (i.e. not sterically hindered), ester group and that the alkyl side groups play a part in determining the odour quality. Since the receptor sites which recognise odorants must have molecular features complementary to the structural features of the odorants, it is inferred that the receptor site(s) recognising esters as fruity-smelling probably possesses a nucleophilic amino acid residue for binding the carbonyl function, and hydrophobic regions for interacting with the alkyl side chains.

Since the function of olfactory receptors is to bind odorant molecules and this activity may be lost during extraction procedures it would be useful to have a method for specifically labelling them *in situ* so that they can be identified during isolation.

Alkyl haloacetates with fruity odours have been shown to be capable of specifically and irreversibly inhibiting EOG responses in a manner most easily explained by an affinity labelling mechanism. Ethyl bromoacetate was found to be particularly effective and may therefore prove to be useful as an affinity labelling odorant enabling olfactory receptors to be identified. It will alkylate nucleophilic amino acid residues and therefore the presence of such a group is indicated in the receptor site(s) with which it interacts.

The use of affinity labelling odorants may provide a means of tagging the chemoreceptive membrane of the olfactory neurons so

that it may be isolated for biochemical studies. Other markers of the olfactory neurons, carnosine* and the olfactory marker protein, are present throughout the length of the olfactory neurons and not specific to the sensory parts, (Margolis and Grillo, 1977, and Graziadei, 1977, respectively).

Experiments were performed in an attempt to clarify the mechanism by which cyclic AMP affects the EOG response. These indicated that if cyclic AMP is involved in the transduction mechanism then odorants cause a decrease in its concentration. An alternative proposal is put forward to explain the means by which the EOG is inhibited by cyclic AMP involving an increased permeability of the supporting cells to chloride ions. This could be tested by examining the effects of cyclic AMP on the summated neural discharge.

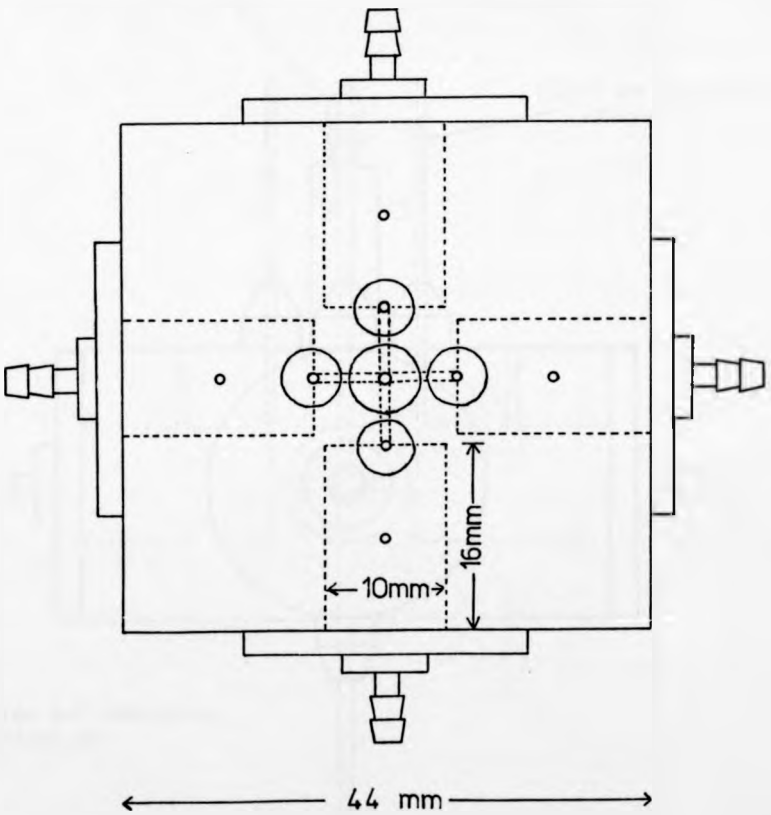
EOG's have been recorded from sheep and bovine olfactory epithelium maintained in vitro. The method of tissue preparation used enabled stable responses to be obtained over a period of several hours. These animals can provide the quantities of tissue that may be necessary for the isolation and identification of receptors and the method of tissue preparation described here will allow electrophysiological experiments to be combined with biochemical studies.

* beta-alanyl-l-histidine

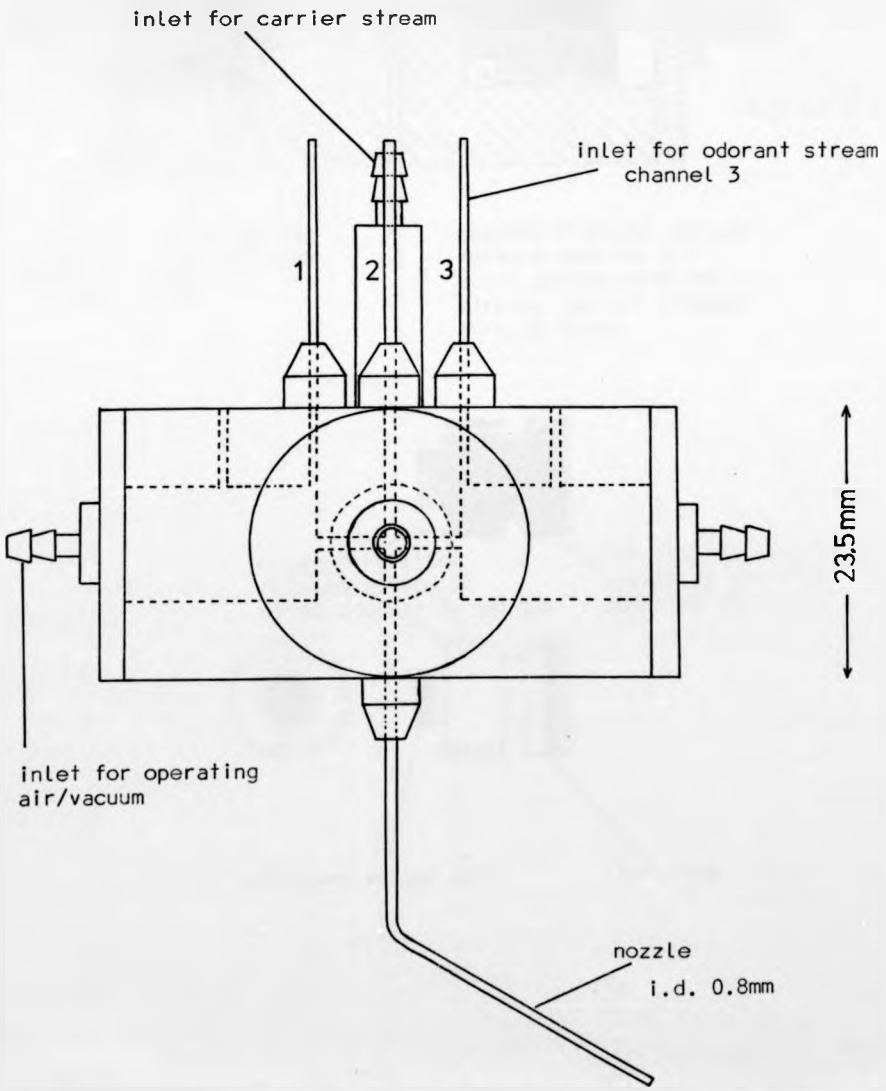
APPENDIX I

Plans of an applicator allowing rapid switching of 1 to 4 channels of odorised air into a carrier stream to produce square-wave pulses of odorant. The construction enables dead-space to be kept to a minimum, (less than 0.01 cm^3), so that the stimulus profile does not become rounded. Odorant streams are injected at 90^o into the carrier stream to ensure mixing.

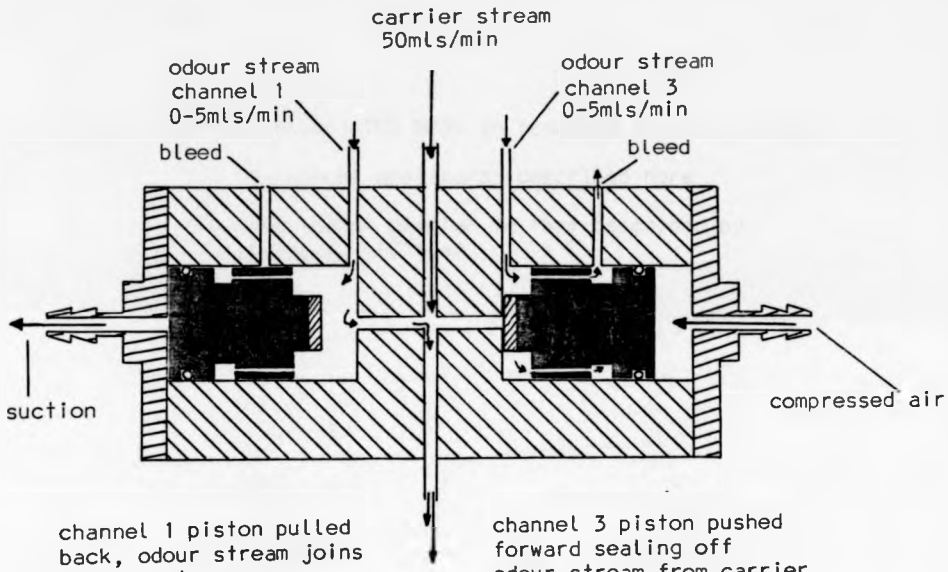
PLAN VIEW OF APPLICATOR SCALE: 2 : 1

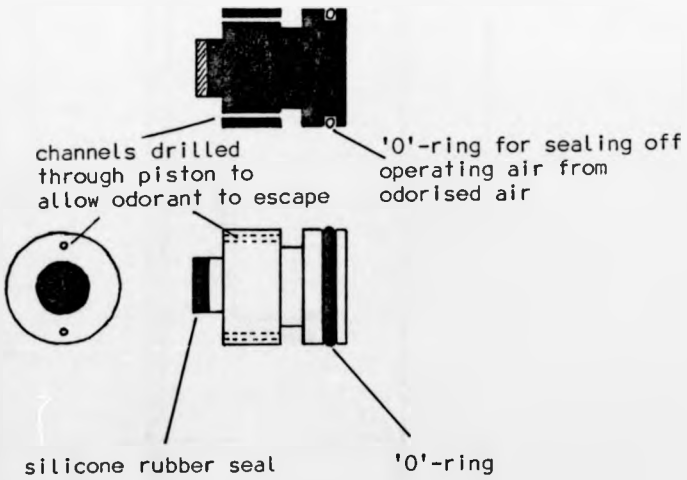


ELEVATED VIEW OF APPLICATOR SCALE: 2 : 1



SWITCHING SYSTEM OF 4-CHANNEL APPLICATOR

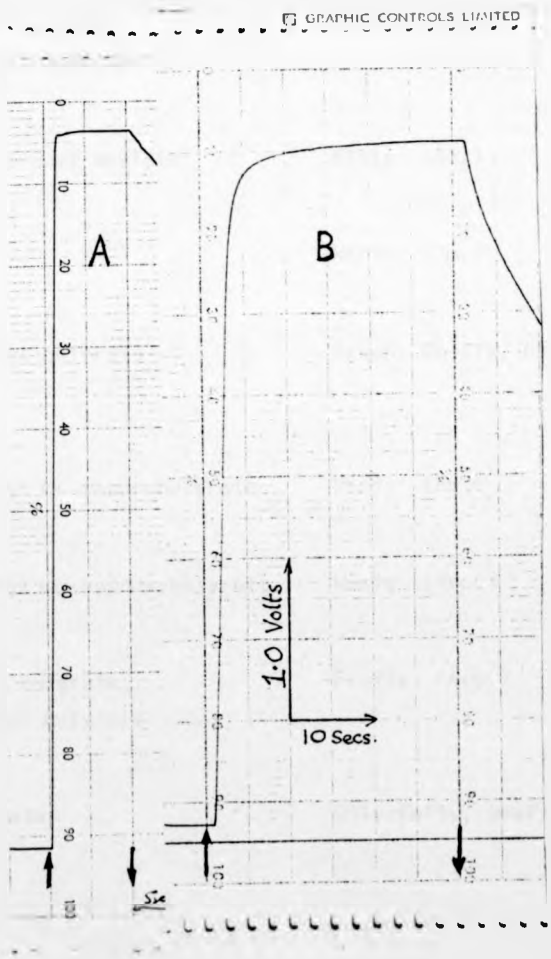




Output from gas sensor (TGS 812) in response to acetone from

(A) 4-channel applicator described here

(B) applicator similar to that described by Poynder, (1974,a)



APPENDIX II

Additional Odour Descriptions

2,2,-dimethyl propyl acetate	Fruity, green (Arc.)
1.1.-dimethyl propyl acetate or t-pentyl acetate	Minty, fruity (Arc.)
1-methyl butyl acetate or sec-pentyl acetate	Fruity (Arc.)
methyl di-iso-propyl acetate	Minty (Am.)
t-butanol	Minty (Am.)
ethyl α-methyl butyrate	Green, fruity, pungent (Arc.)
ethyl α-methyl α-chlorobutyrate	Minty (Am.)
ethyl α-methyl α-hydroxybutyrate	Minty (Arc.)
1-methyl butyl butyrate or sec-pentyl butyrate	Fruity (Arc.)
ethyl dodecanoate	Oily-fatty, leafy, fruity (Arc.)

t-butyl ethyl ether or pinacolone	Ethereal, minty, camphoraceous (Arc.)
t-butyl methyl ether	Minty, camphoraceous (Am.)
butyl heptanoate	Fruity, green (Arc.)
iso-butyl heptanoate	Fruity-winey, green (Arc.)
ethyl heptanoate	Fruity-winey (Arc.)
ethyl hexadecanoate	Fruity, waxy, sweet (Arc.)
ethyl hexanoate	Fruity-winey (Arc.)
ethyl nonanoate	Fatty-oily, fruity, nutty winey (Arc.)
ethyl octanoate	Fruity-winey (Arc.)
ethyl pentadecanoate	Sweet, musky (Arc.)
ethyl α-chloropentanoate	Minty (Am.)
ethyl α-chloro-iso-pentanoate	Minty (Am.)

REFERENCES

(Arc.) Arctander, (1969)

(Am.) Amoore, (1970)

APPENDIX III

(a) Evidence for the electrical responses produced by *in vitro* mammalian olfactory epithelium in response to odorous stimulation being EOG's and representing the summated generator potential of the olfactory neurons.

i) No responses were elicited from control tissues, (brain, liver, lamina propria)

ii) The responses had typical EOG shapes,(Figure (A))

iii) The responses were concentration dependent, (Figure (B))

iv) The responses showed adaptation

v) The rise time of the response decreased with increasing odorant concentration, as was found by Ottoson, (1956), for frog EOG's

vi) The responses were similar to those obtained by Gesteland and Sigwart, (1977), and Poynder *et al*, (1978), who also recorded single unit responses

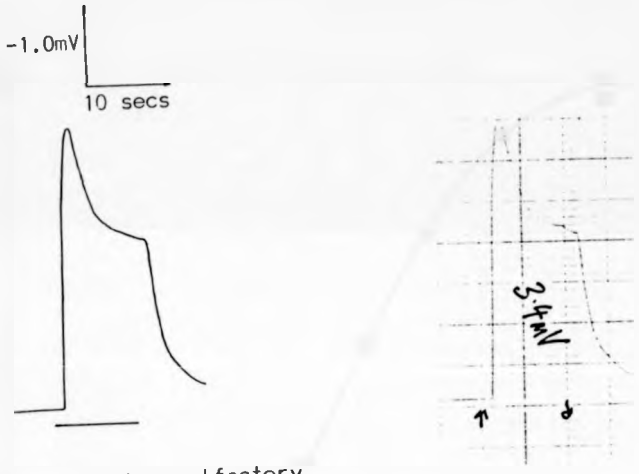
vii) Responses from frog olfactory epithelium *in vitro* were the same as *in vivo*, (Figure (A))

viii) There was a spatial distribution in the sensitivity of the epithelium to odorants, as has been found in the frog, (e.g Mustaparta, 1971), (Figure (H))

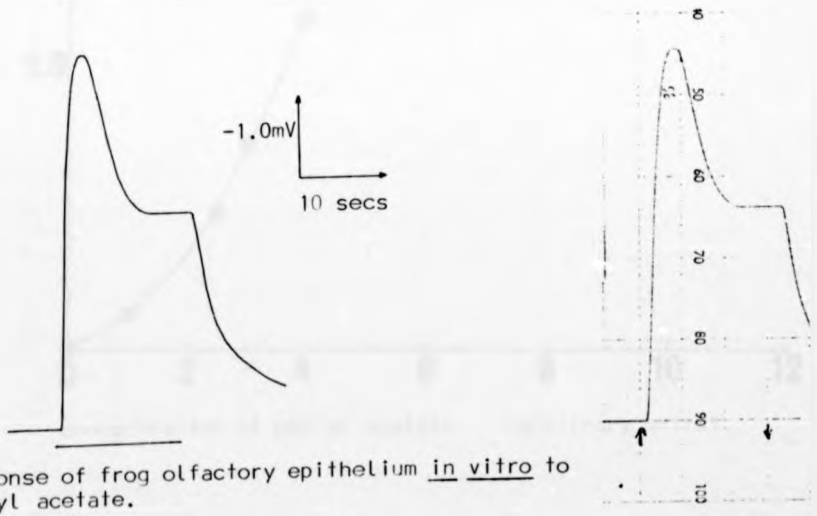
ix) The response was temperature dependent, (Figures (C) to (G))

FIGURE (A)

EOG's recorded from sheep and frog olfactory epithelium *in vitro*



Response of sheep olfactory epithelium to pentyl acetate
Compare with Figure (49), page 118



Response of frog olfactory epithelium *in vitro* to pentyl acetate.

FIGURE (B)

Concentration dependence of responses from sheep olfactory epithelium *in vitro* to pentyl acetate

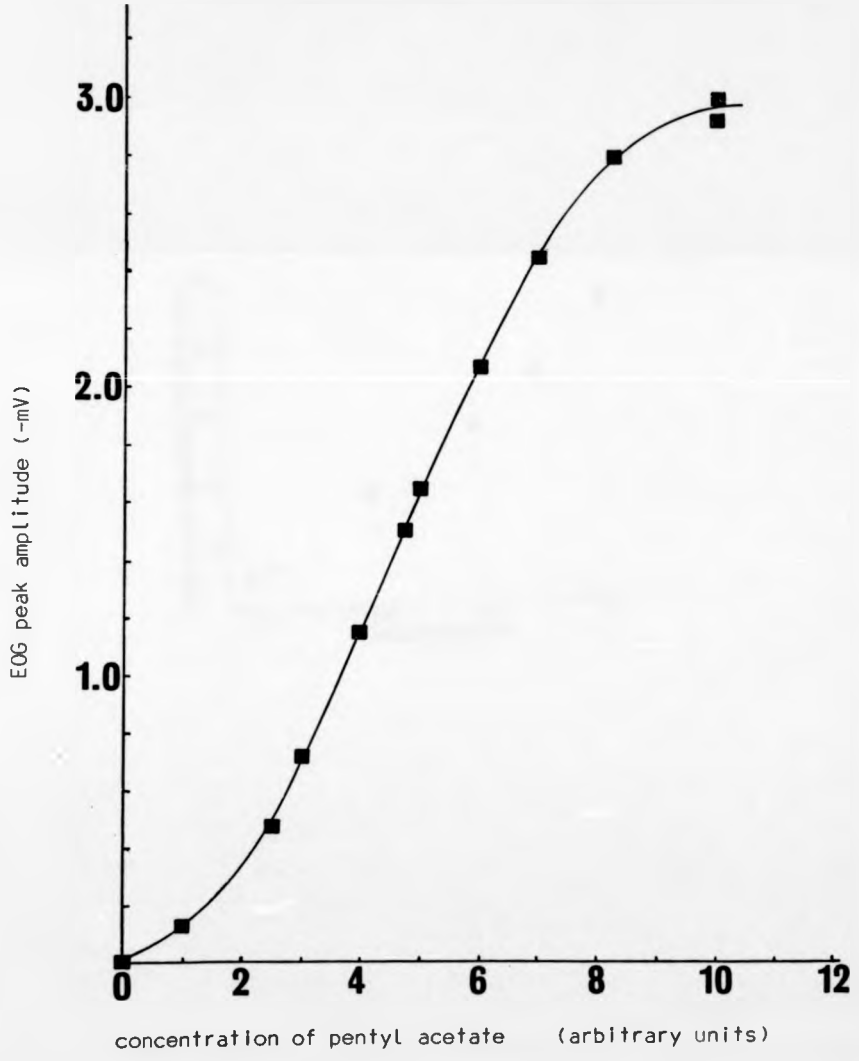


FIGURE (C)

Temperature dependence of the generator potential in the Pacinian corpuscle, from Loewenstein, (1965)

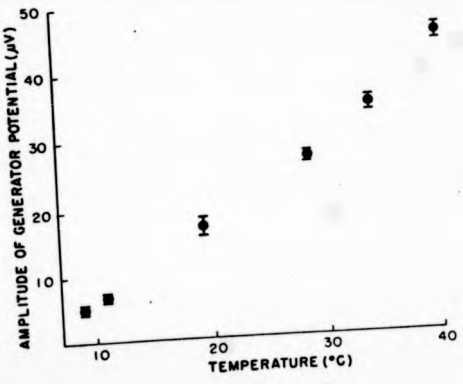


FIGURE (D)

Sheep olfactory epithelium *in vitro*: changes in EOG peak amplitude to pentyl acetate with increasing tissue temperature.

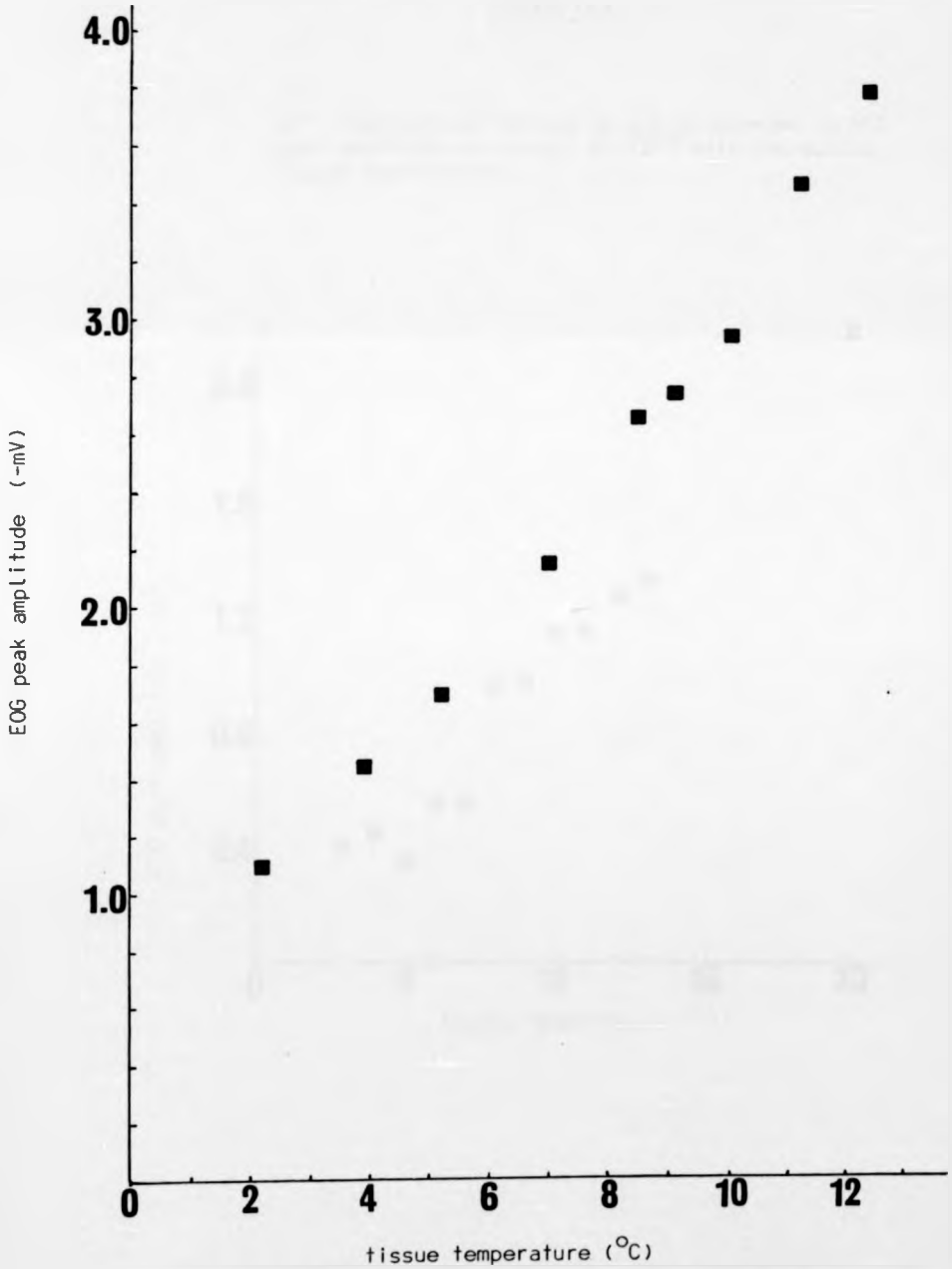


FIGURE (E)

Rat olfactory epithelium *in vitro*: changes in EOG peak amplitude to pentyl acetate with increasing tissue temperature.

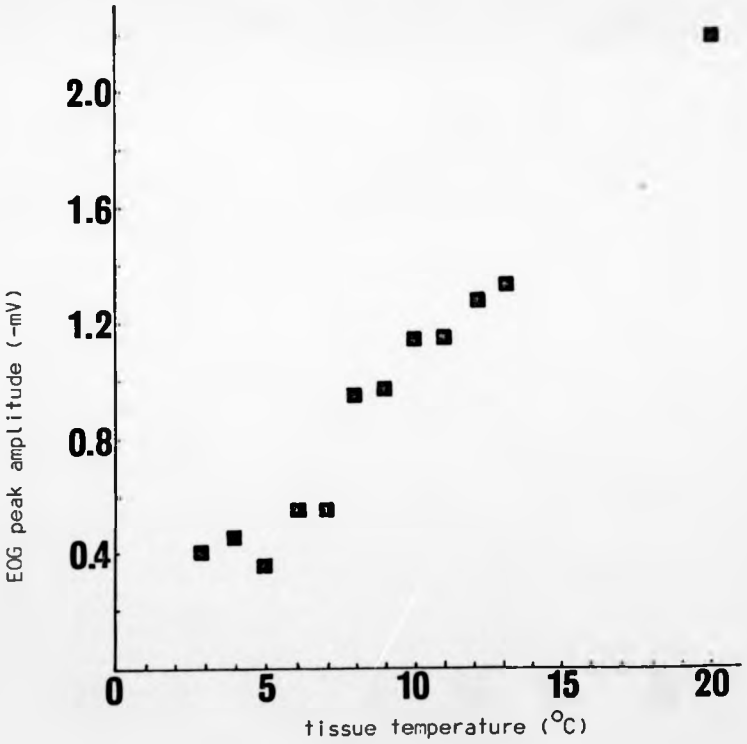


FIGURE (F)

Calf olfactory epithelium *in vitro*: changes in EOG peak amplitude to pentyl acetate with increasing tissue temperature.

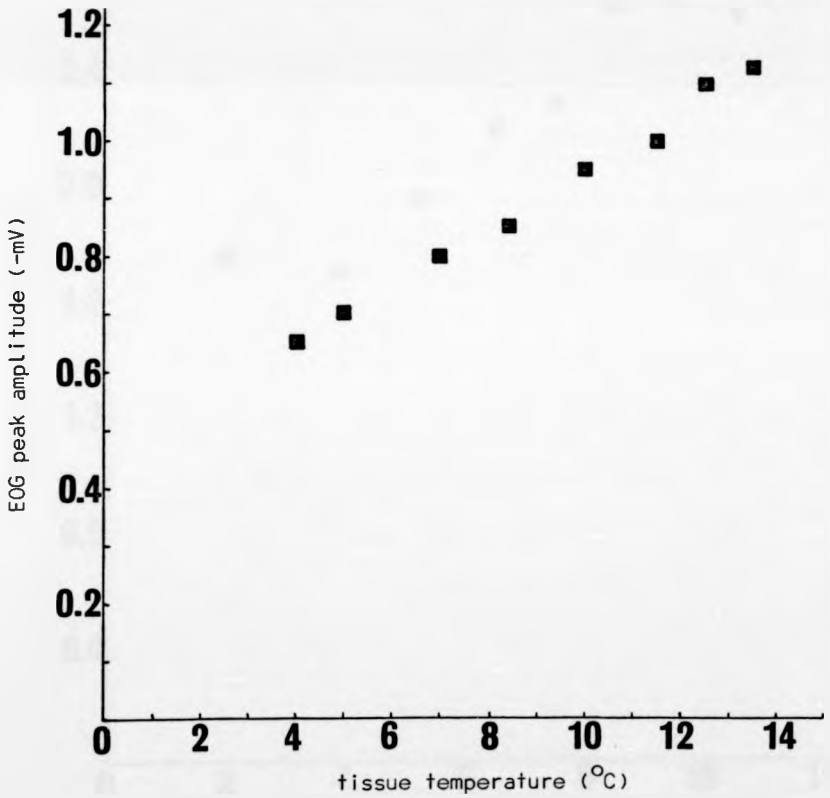


FIGURE (G)

Frog olfactory epithelium *in vitro*: changes in EOG peak amplitude to pentyl acetate with increasing tissue temperature.

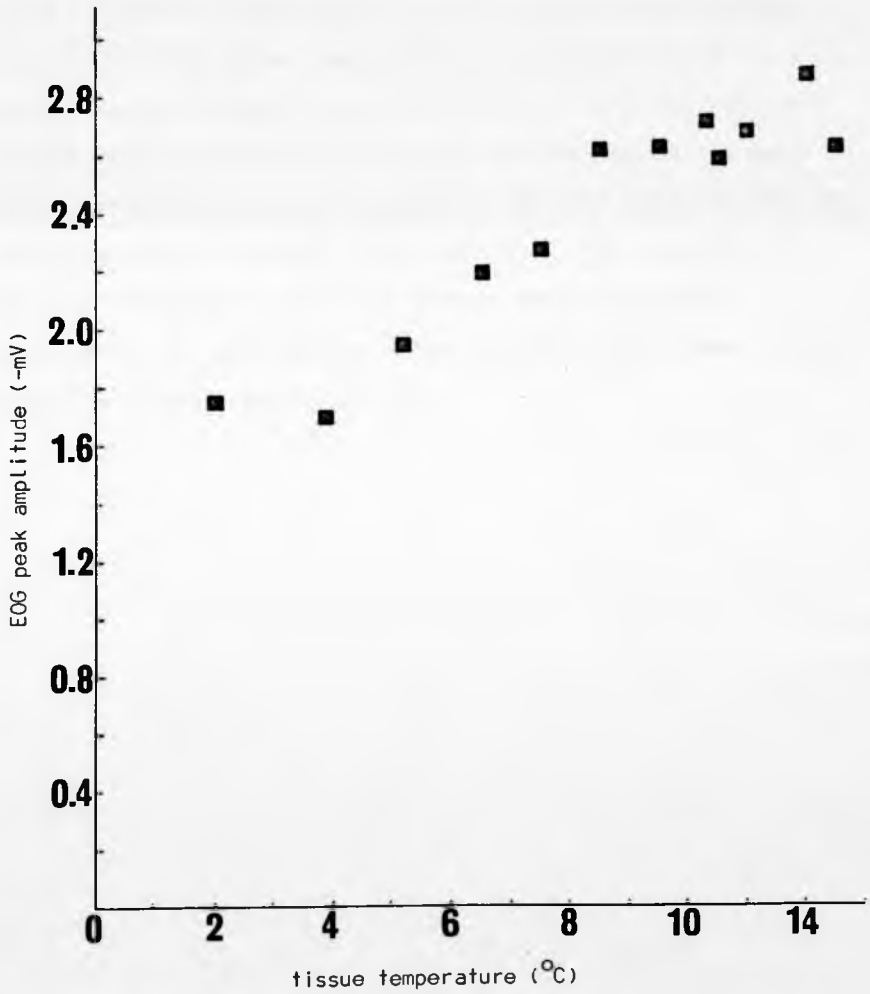
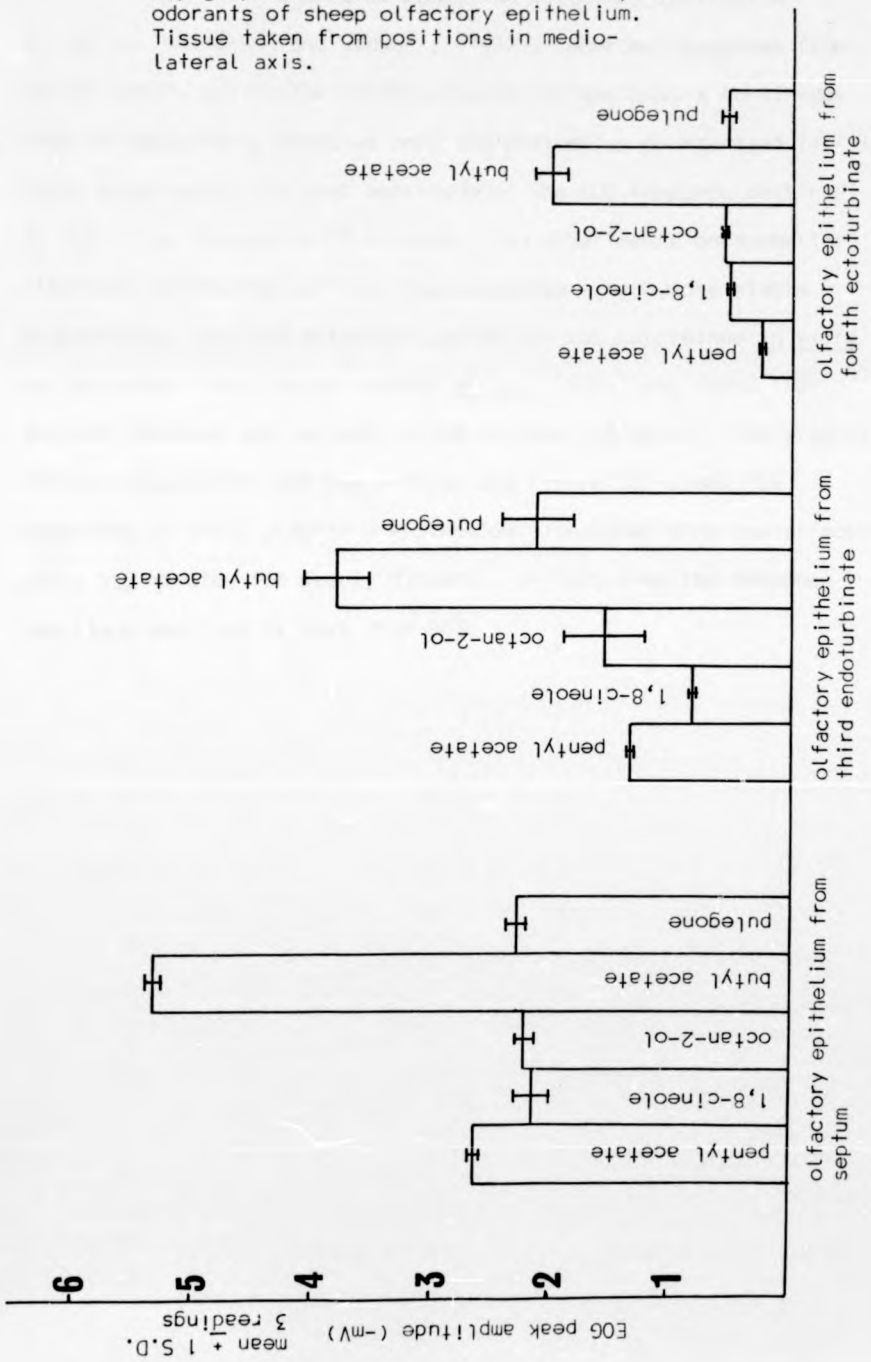


Figure (H) - topographical variation in sensitivity to odorants of sheep olfactory epithelium. Tissue taken from three positions in medio-lateral axis on right hand side of head. Tissue taken from septum (most medial), medial face of 3rd endoturbinate, and medial face of 4th ectoturbinate (most lateral). Largest responses obtained from septal olfactory epithelium where density of olfactory neurons is highest, smallest responses obtained from tissue taken from most lateral position. Both absolute and relative EOG response amplitudes varied. Same flow rate of odorised air for each channel; tissue from one animal; mean of three responses for each odorant $\pm$ 1 S.D.

FIGURE (H)

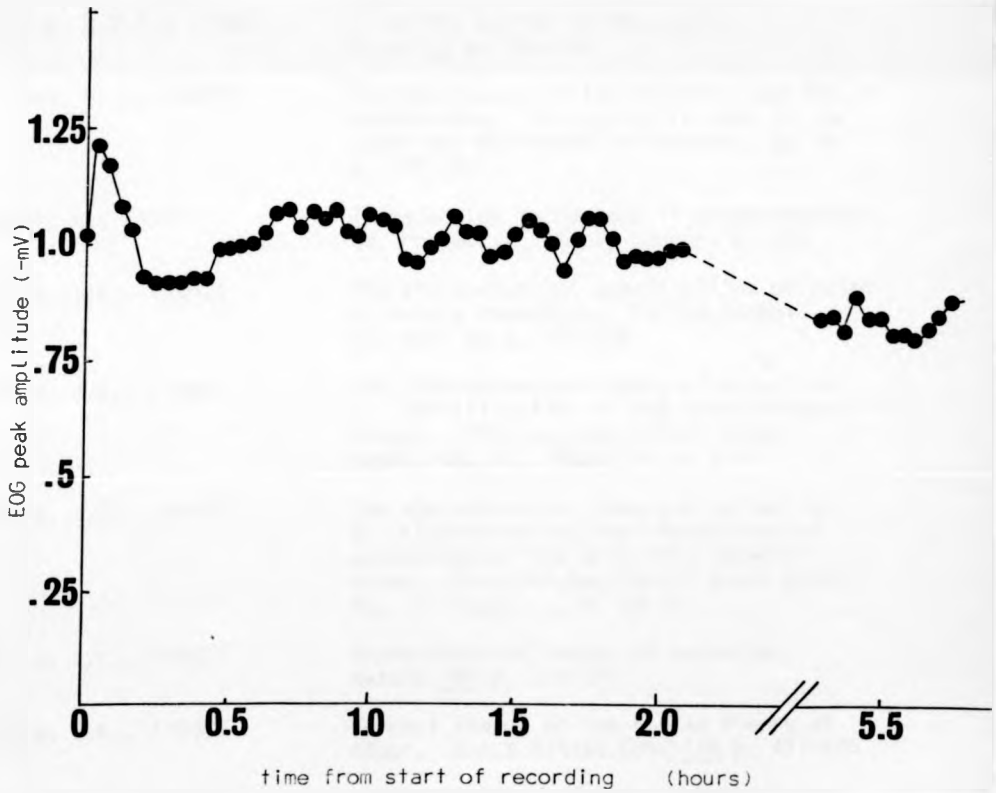
Topographical variation in sensitivity to odorants of sheep olfactory epithelium. Tissue taken from positions in medio-lateral axis.



(b) Maintenance of mammalian olfactory epithelium *in vitro*. Gesteland and Sigwart, (1977), recorded responses from the olfactory epithelium of decapitated rat and used a continuous flow of $95\%O_2/5\%CO_2$ directed onto the epithelium to maintain it. In their experiments the peak amplitude of the EOG response declined by 50% in an average of 90 minutes. The experiments on mammalian olfactory epithelium in this study required a much more stable preparation. Excised olfactory epithelium was maintained *in vitro* in the manner reported by Poynder *et al*, (1978), who found that the EOG response was reduced by 50% in about 24 hours. The stability of the preparation here was similar and Figure (J) shows the responses of sheep olfactory epithelium stimulated with pentyl acetate every 2.5 minutes for nearly 6 hours. In this time the response amplitude declined by less than 20%.

FIGURE (J)

Stability of the EOG response from sheep olfactory epithelium maintained in vitro. EOG's to pentyl acetate recorded at 2.5 minute intervals for 340 minutes. Tissue from septum; electrode position kept constant.



REFERENCES

Abramson, M., and Harker, L.A., (1973) — Physiology of the nose. Otolaryngol. Clin. N. Am. 6 p. 623-635

Adrian, E.D., (1953) — Sensory messages and sensation. The response of the olfactory organ to different smells. Acta. Physiol.Scand. 29 p. 4-14

Alcock, N., (1901) — The histology of the nasal mucus membrane of the pig. Anat.Record 4 p. 123-138

Allison, A.C., and Warwick, R.T.T., (1949) — Quantitative observations on the olfactory system of the rabbit. Brain 72 p. 186-197

Allison, A.C., (1953) — The morphology of the olfactory system in vertebrates. Biological reviews of the Cambridge Philosophical Society, 28 (2) p. 159-244

Altner, H., (1974) — Transduction mechanisms in Chemoreception, ed. Poynder. I.R.L., London. p. 291

Amoore, J.E., (1952) — The stereochemical specificities of human olfactory receptors. Perfum.Essent. Oil Rec. 43 p. 321-330

Amoore, J.E., (1962) — The stereochemical theory of olfaction. 1 - Identification of the seven primary odours. Proc.Sci.Sec.Toilet Goods Assoc. No. 37, (Suppl.), p. 1-12

Amoore, J.E., (1962) — The stereochemical theory of olfaction. 2 - Elucidation of the stereochemical properties of the olfactory receptor sites. Proc.Sci.Sec.Toilet Goods Assoc. No. 37, (Suppl.), p. 13-23

Amoore, J.E., (1963) — Stereochemical theory of olfaction. Nature 198 p. 271-271

Amoore, J.E., (1964) — Current status of the steric theory of odour. Ann.N.Y.Acad.Sci. 116 p. 457-476

Amoore, J.E., (1970) — Molecular Basis of Odour. Publ. C. C. Thomas, Illinois

Arctander, S., (1969) — Perfume and Flavour Chemicals. Publ. Arctander,(2 volumes).

Bannister, L., (1974) — Possible functions of mucus at gustatory and olfactory surfaces. Transduction Mechanisms in Chemoreception. ed. Poynder, I.R.L., London. p.39-48

Barnard, E.A., and Stein, W.D., (1959) — The histidine residue in the active centre of ribonuclease, (1) - a specific reaction with bromoacetic acid. J.Mol.Biol. 1 p. 339-349

Beam, K.G., and Greengard, P., (1978) — Cyclic nucleotides, protein phosphorylation and synaptic function. Cold Spring Harb. Symp. Quant. Biol. XL p. 157-168

Bedini et al, (1976) — Bedini, C., Fiaschi, A., and Lanfranchi, A., Degenerative and regenerative processes in the olfactory system of homing pigeons. Arch. Ital. Biol. 114 p. 376-388

Bhatnagar, K.P., and Kallen, F.C., (1975) — Acta Anat. 91 p. 272 quoted from van Drongelen et al, (1978)

Bitensky et al, (1972) — Bitensky, M.W., Miller, W.H., Gorman, E.R., Neufeld, A.H., and Robinson, R., The role of cyclic AMP in visual excitation Adv. in Cyclic Nuc. Res. 1 p. 317-335

Boelens, V.H., and Heydel, J., (1973) — " Eine studie uber strukturelle eigenschaften chemischer verbindungen mit verschiedenen geruchscharakteristiken. Chemiker-Zeitung 97 (1) p. 8-15

Bostock, H., (1974,a) — Diffusion and the frog EOG. Transduction Mechanisms in Chemoreception, ed. Poynder, I.R.L. Ltd., London. p. 27-38

Bostock, H., (1974,b) — An Analysis of the Frog Electro-olfactogram PhD Thesis, University of London

von Braun et al, (1929) — " von Braun, J., Kraper, H., and Wienhaus, H., Odour and constitution. Ber. 62, (B), p. 2,880-2,885

Brower, K.R., and Schafer, R., (1975) — Recognition of chemical types by odour: effect of steric hindrance at the functional group. J.Chem. Ed. 52, (8), p. 538-540

Byzov, A.L., and Flerova, G.I, (1964) — Electrophysiological investigation of frog olfactory epithelium. Biofisika 9, p. 217-225, (in Russian)

Chasin, M., and Harris, D.N., (1972) — Cyclic 3',5' - nucleotide phosphodiesterase. Methods in Mol. Biol. 3 p. 169-198

Costanzo, R.M., and Mozell, M.M., (1976) — Electrophysiological evidence for a topographical projection of the nasal mucosa onto the olfactory bulb of the frog. J. Gen. Physiol. 68 p. 297-312

Costanzo, R.M., and O'Connell, R.J., (1978) — Spatially organised projection of hamster olfactory nerves, Brain Res. 139 p. 327-332

Daval, G., and Leveteau, J., (1969) — Analyse topographique de l'electro-olfactogramme chez la grenouille. J. Physiol. (Paris), 61 suppl. (2) p.257-258

Daval, et al, (1972) — Daval, G., Leveteau, J., MacLeod, P., Holley, A., Duchamp, A., and Revial, M.F., Studies on local EOG and single receptor responses to multiple odour stimulation in the frog. Olfaction and Taste IV ed. Schneider, W.V.G., Stuttgart, p. 109-120

Dodd, G.H., (1976) — Ligand-binding phenomena in chemo-reception. Structure-Activity Relationships in Chemoreception. Ed. G. Benz., I.R.L., London. p.55-61

Døving, K., (1966) — Problems in the physiology of olfaction. Chemistry and Physiology of Flavours ed. Schultz et al, Avi Publ. Comp. Conn. p. 52-94

Døving, K., and Belghaug, R., (1977) — Frequency of induced waves in the olfactory bulb in char (Salmo alpinus L); a temperature dependence. Comp.Biochem. Physiol. 56A p. 577-579

van Drongelen, W., (1978) — Unitary recordings of near threshold responses of receptor cells in the olfactory mucosa of the frog. J.Physiol. 277 p. 423-435

van Drongelen et al, (1978) — van Drongelen, W., Holley, A., and Døving, K.B., Convergence in the olfactory system: quantitative aspects of odour sensitivity. J. Theor Biol. 71 p. 39-48

Duchamp et al, (1974) — Duchamp, A., Revial, M.F., Holley, A., and MacLeod, P., Odour discrimination by frog olfactory receptors. Chem. Sens. and Flav. 1 p. 213-233

Exton et al, (1972) — Exton, J.H., Lewis, S.B., Ho, R.J., and Park, C.R., The role of cyclic AMP in the control of hepatic glucose production by glucagon and insulin. Adv. Cyclic Nuc. Res. 1 p. 91-101

Eyzaguirre, C., and Kuffler, S.W., (1955) — Processes of excitation in the dendrites and in the soma of single isolated sensory nerve cells of the lobster and crayfish. J. Gen. Physiol. 39 p. 87-119

Fager et al, (1973) — Fager, R.S., Kutina, C.B., Abrahamson, E.W., The use of NBD-chloride (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) in detecting amino acids and as an N-terminal reagent. Analyt. Biochem. 53 p. 290-294

Ferron, J, (1973) — Morphologie comparee de l'organe de l'odorat chez quelques mammiferes carnivores. Naturaliste Canad., 100 p. 525-541

Fesenko et al, (1977) — Fesenko, E.E., Novoselov,V.I., Pervukhin, G.Y., and Fesenko, N.K. Molecular mechanisms of odour-sensing 11. Studies of fractions from olfactory tissue scrapings capable of sensitizing artificial lipid membranes to action of odorants. Biochim. Biophys. Acta 466 p. 347-356

Filburn et al, (1978) — Filburn, C.R., Colpo, F., and Sacktor, B. Regulation of cyclic nucleotide phosphodiesterase of cerebral cortex by Ca^{++} and cyclic GMP. J. Neurochem. 30 p. 337-346

Freeman, W.J., (1978) — Spatial properties of an EEG event in the olfactory bulb and cortex. Electroencephalography and Clinical Neurophysiology 44 p. 586-605

Friedman, L., and Miller, J.G., (1971) — Odour incongruity and chirality. Science 172 p. 1044-1046

Fuortes, M.G.F.,(1971) — Generation of responses in receptor. Handbook of Sensory Physiology 1, Principles of Receptor Physiology, ed. Loewenstein, Springer-Verlag, Berlin. p. 243-268

Gasser, S.H., (1956) — Olfactory nerve fibres. J.Gen. Physiol 39 (4) p. 473-496

Gemne, G., and Døving, K.B., (1969) — Amer. J. Anat. 126 p. 457 quoted from van Drongelen et al, (1978)

Gesteland, R.C., (1964) — Initial events of the electro-olfactogram Ann. N.Y. Acad.Sci. 116 p. 440-447

Gesteland, R.C., (1971) — Neural coding in olfactory receptor cells. Handbook of Sens. Physiol. IV. Chemical Senses (1) Olfaction ed. Beidler, Springer-Verlag, Berlin. p. 132-150

Gesteland, R.C., and Sigwart, C.D., (1977) — Olfactory receptor units: a mammalian preparation. Brain Research, 133 (7977) p. 144-149

Gesteland et al, (1963) — Gesteland, R.C., Lettvin, J.Y., Pitts, W.H., and Rojas, A. Odour specificities of the frog's olfactory receptors. Olfaction and Taste I, ed. Zotterman, Pergamon Press. p. 19-44

Gesteland et al, (1965) — Gesteland, R.C., Lettvin, J.Y., and Pitts, W.H. Chemical transmission in the nose of the frog. J. Physiol. (London) 181 p. 525-559

Getchell, M.L., (1971) — The nature of the receptor sites in the frog's olfactory epithelium. PhD thesis, Northwestern University, U.S.A

Getchell, M.L. and Gesteland, R.C., (1972) — The chemistry of olfactory reception: stimulus-specific protection from sulphydryl reagent inhibition. Proc. Nat. Acad. Sci. 69 (6), p. 1494-1498

Ghosh, P.B., and Whitehouse, M.W., (1968) — 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole: a new fluorigenic reagent for amino acids and other amines. Biochem. J. 108 p. 155-156

Graziadei et al, (1977) — Graziadei, G.A.M., Margolis, F.L., Harding, J.W., and Graziadei, P.P.C. Immunocytochemistry of the olfactory marker protein.J.Histochem. and Cytochem. 25, (12), p. 1311-1316

Graziadei, P.P.C., (1966) — Electron microscopic observations of the olfactory mucosa of the mole. J.Zool. London 149 p. 89-94

Graziadei, P.P.C., (1971) — The olfactory mucosa of vertebrates. Handbook of Sens. Physiol. IV Chem. Sens. 1 - Olfaction, ed. Beidler, Springer-Verlag, Berlin. p. 27-58

Graziadei, P.P.C., (1972) — Functional anatomy of vertebrates olfactory mucosa. Olfaction and Taste IV, p. 13-19

Graziadei, P.P.C., (1973) — Cell dynamics in the olfactory mucosa. Tissue and Cell 5 (1) p. 113-131

Graziadei, P.P.C., (1978) — The olfactory sensory neuron as a model for the study of regeneration. Abstracts 3rd ECRO Congress, Pavia. p. 60

Graziadei, P.P.C., and Metcalf, J.F., (1971) — Autoradiographic and ultrastructural observations on the frog's olfactory mucosa. Z.Zellforsch 116 p. 305-318

Greengard, P., (1978) — Phosphorylated proteins as physiological effectors. Science 199 p. 146-152

Harper et al, (1968) — Harper, R., Bate-Smith, E.C., Land, D.G., and Griffiths, N.M. A glossary of odour stimuli and their qualities. Perfum. Essent. Oil Rec. 59 p. 22-37

Heist et al, (1967) — Heist, H.E., Mulvaney, B.D., and Landis, D.J., Odour sensing cell ultra structure by electron microscopy. Honeywell Inc. Corporate Res. Centre, Hopkins, Minnesota, U.S.A.

Hendrickson et al, (1970) — Hendrickson, J.B., Cram, D.J., and Hammond, G.S. Organic Chemistry p. 395-397 McGraw-Hill Book Co.

Henning, H., (1915) — Der Geruch. I.Z. Phychol. 73 p. 161-257

Holley, A., (1974) — The discriminating power of the vertebrate olfactory receptors. Transduction Mechanisms in chemo-reception, ed. Poynder, I.R.L., London. p. 275-289

Holley, A., and Døving,K.B., (1978) — Receptor sensitivity acceptor distribution convergence and neural coding in the olfactory system. Olfaction and Taste VI., I.R.L., London

Hornung, D.E., and Mozell, M.M., (1977) — Factors in influencing the differential sorption of odorant molecules across the olfactory mucosa. J.Gen.Physiol. 69 p. 343-361

Hosoya, Y., and Yoshida, H., (1937) — Uber die bioelektrischen erscheinungen an der reichschleimhaut Jap J. Med. Sci.III Biophysics 5 p. 22-23

Jakoby, W.B., and Wilchek, M, (eds.), (1977) — Affinity Labelling. Methods in Enzymology Vol. XLVI, Academic Press

Kanda et al, (1973) — Kanda, T., Kitamura, T., Kaneko, T., and Scanning electron microscopic observations on the olfactory epithelium of guinea-pig and human being. J.Otolaryngol. Japan, 76 p. 30 (in Japanese, quoted from Menco, 1977)

Kauer, J.S., and Moulton, D.G., (1974) — Responses of olfactory bulb neurons to odour stimulation of small nasal areas in the salamander. J. Physiol. 243 p. 717-737

Kimura, K., (1961) — Olfactory nerve responses of frog. Kumamoto Med. J., 14 p. 37-46

Korman, S., and Clarke, H.T. (1956) — Carboxymethyl amino acids and peptides. J. Biol. Chem. 221 p. 113-131

Kramer, G., (1933) — Untersuchungen über die sinnesleistungen und das orientierungsverhalten von Xenopus laevis,DAUD.Zool.Jahrb.Physiol. 52 p. 629-676

Kratzing, J.E., (1970) — The olfactory mucosa of the sheep. Aust. J. Biol. Sci. 23 p.447-458

Kurihara, K, and Koyama, N., (1972) — High activity of adenyl cyclase in olfactory and gustatory organs. Biochem. and Biophys. Res. Comm. 48 (1) p. 30-34

Laffort, P., (1963) — Essai de standardization des sevils olfactifs humains pour 192 corps purs. Arch. Sci. Physiol. 17 p. 75-105

Lehninger, A.L., (1970) — Biochemistry. Worth Publishers Inc., New York. p. 171

Loewenstein, W.R., (1965) — Facets of a transducer process. Cold Spring Harb. Symp. Quant. Biol. XXX p. 29-44

de Lorenzo, A.J.D., (1970) — The olfactory neuron and the blood-brain barrier. Taste and Smell in Vertebrates ed. Wolstenholme and Knight, J.& A Churchill, London. p. 151-173

MacLeod, P., (1959) — Premiere donnes sur l'electro-olfactogramme du lapin. J. Physiol (Paris) 51 p. 85-92

Margolis, F.L., and Grillo, M., (1977) — Axoplasmic transport of carnosine in the mouse olfactory pathway. Neurochem. Res. 2 p. 507-519

Mathews, D.F., (1972) — Olfactory response patterns in the tortoise. J. Gen. Physiol. 60 p. 166- 180

Matthes, E. (1927) — Z.verg;.Physiol. (1927) 5 p. 83, (from Ottoson, 1956)

Matulionis, D.H., (1975) — Ultrastructural study of mouse olfactory epithelium following destruction by $ZnSO_4$ and its subsequent regeneration. Am. J. Anat. 142 (1) p. 67-90

Menco, B.P.M., (1977) — PhD thesis. A qualitative and quantitative investigation of olfactory and nasal respiratory mucosal surfaces of cow, and sheep based on various ultra-structural and biochemical methods. H. Veenman and Zonen B.V-Wageningen, Holland.

Menco, B.P.M., (1978) — Freeze-fracture studies on olfactory and respiratory cilia and microvilli in frog, rat and dog. Abstracts 3rd ECRO Congress Pavia

Menco *et al*, (1976) — Menco, B.P.M., Dodd, G.H., Davey, M., and Bannister, L.H. Presence of membrane particles in freeze-etched bovine olfactory cilia. Nature 263 p. 597-599

Menevse, A., (1973) — MSc. Thesis, University of Warwick

Menevse, A., (1977) — PhD Thesis , University of Warwick

Menevse *et al*, (1976) — Menevse,A., Squirrell, D.J., Dodd, G.H., and Poynder, T.M., A chemical modification approach to the olfactory code. Abstracts 2nd ECRO Congress, Reading p. 73

Menevse *et al*, (1977,a) — Menevse, A., Dodd, G.H., Poynder, T.M., and Squirrell, D.J., A chemical modification approach to the olfactory code. Biochem. Soc. Trans. 5 p. 191-194

Menevse *et al*, (1977,b) — Menevse, A., Dodd, G.H., and Poynder, T.M. Evidence for the specific involvement of cyclic AMP in the olfactory transduction mechanism. Biochem, and Biophysiol. Res. Comm. 77 (2) p. 671-677

Minor, A.V., and Sakina, N.L., (1973) — The role of cyclic adenosine 3',5'-monophosphate in olfactory reception. Neirofiziologia 5 (5), p. 414-422 (in Russian)

Moncrieff, R.W., (1951) — The Chemical Senses. Leonard Hill Ltd., London

Moulton, D.G., (1965) — Differential sensitivity to odours. Cold Spring Harb. Symp. Quant. Biol. XXX p. 201-206

Moulton, D.G., (1967) — Spatio-temporal patterning of response in the olfactory system. Olfaction and Taste II, ed. Hayashi, Pergamon Press, p. 109-116

Moulton, D.G., (1975) — Cell renewal in the olfactory epithelium of the mouse. Olfaction and Taste V, ed. Denton and Coghlan, Academic Press Inc., New York.

Moulton, D.G., (1976) — Spatial patterning of response to odours in the peripheral olfactory system. Physiol. Rev. 56 (3) p. 578-593

Moulton, D.G., and Fink, R.P., (1972) — Cell proliferation and migration in the olfactory epithelium. Olfaction and Taste IV ed. Schneider. Wissenschaftliche Verlagsgesellschaft MBH, Stuttgart, p. 20-26

Moulton et al, (1970) — Moulton, D.G., Celebi, G., and Fink, R.P. Olfaction in mammals - two aspects: proliferation of cells in the olfactory epithelium and sensitivity to odours. Taste and Smell in Vertebrates ed. Wolstenholme and Knight, J & A Churchill, London. p. 227-250

Mozell, M.M., (1962) — Olfactory mucosal and neural responses in the frog. Am. J. Physiol. 203 p. 353-358

Mozell, M.M., (1964,a) — Olfactory discrimination: electrophysiological spatio-temporal basis. Science 143 p. 1336-1337

Mozell, M.M., (1964,b) — Evidence for sorption as a mechanism of the olfactory analysis of vapours. Nature, 203 p. 1181

Mozell, M.M., (1966) — The spatio-temporal analysis of odorants at the level of the olfactory receptor sheet. J. Gen. Physiol. 50 p. 25-41

Mozell, M.M., (1967) — The effect of concentration upon the spatio-temporal coding of odorants. Olfaction and Taste II ed. Hayashi, Pergamon Press p. 117-124

Mozell, M.M., (1969) — Migration of odorant molecules. Olfaction and Taste III ed. Pfaffman Rockefeller Press, New York City p. 221-225

Mozell, M.M., (1970) — Evidence for a chromatographic model of olfaction. J.Gen. Physiol. 56 p. 46-63

Mozell, M.M., (1971) — Spatial and temporal patterning. Handbook of Sens. Physiol. IV Chem. Sens. I, Olfaction ed.Beidler, Springer-Verlag, Berlin. p. 205-215

Mozell, M.M., and Jagodowicz, M., (1973) — Chromatographic separation of odorants by the nose: retention measured across in vivo olfactory mucosa. Science 181 p. 1247-1249

Müller, A., (1953) — Quantitative untersuchungen am reichepithel des hundes. Z.Zellforsch 41 p. 335-350

Muller, W., and Kiepenhever, J., (1976) — Olfactory orientation in frog. Naturwissenschaft 57 (1) p. 23-28

Mulvaney, B.D., and Heist, H.E., (1970) — Mapping of rabbit olfactory cells. J. Anat. 107 p. 19-30

Mustaparta, H. , (1971) — Spatial distribution of receptor responses to stimulation with different odours. Acta Physiol. Scand. 82 p. 154-166

Negus, V., (1958) — The comparative anatomy and physiology of the nose and paranasal sinuses. Livingstone, Edinburgh. (From Graziadei, 1971)

O'Connell, R.J., and Mozell, M.M., (1969) — Quantitative stimulation of frog olfactory receptors. J. Neurophysiol. 32 p. 51-63

Okano, M., and Takagi, S.F., (1974) — Secretion and electrogenesis of the supporting cell in the olfactory epithelium. J. Physiol. 242 p. 353-370

Okano et al, (1967) — Okano, M., Weber, A.F., and Frommes, S.P., Electron microscopic studies of the distal border of canine olfactory epithelium. J. Ultrastruct.Res. 17 p. 487-502

Osterhammel et al, (1969) — Osterhammel, P., Terkildsen, K., and Zilstoff, K. Electro-olfactograms in man. J. Laryngol and Otolaryngol 83 p. 731-735

Ottoson, D., (1954) — Sustained potentials evoked by olfactory stimulation. Acta Physiol.Scand. 32 p. 384-386

Ottoson, D., (1956) — Analysis of the electrical activity of the olfactory epithelium. Acta Physiol.Scand. 35 (suppl. 122), p. 1-83

Ottoson, D., (1959) — Comparison of slow potentials evoked in the frog's nasal mucosa and olfactory bulb by natural stimulation. Acta Physiol. Scand. 47 p. 149-159

Ottoson, D., (1971) — The electro-olfactogram. Handbook of Sens. Physiol. IV Chemical Senses 1, Olfaction p. 95-131

Ottoson, D., (1974) — Generator potentials. Transduction Mechanisms in Chemoreception, ed. Poynder, I.R.L., London. p. 231-239

Ottoson, D., and Shepherd, G.M., (1965) — Receptor potentials and impulse generation in the isolated spindle during controlled extension. Cold Spring Harb. Symp. Quant. Biol. 30 p. 105-114

Ottoson, D., and Shepherd, G.M., (1967) — Experiments and concepts in olfactory physiology. Sensory Mechansims: Progress in Brain Research, ed. Zotterman, Elsevier, Amsterdam. p. 83-138

Parke, D.V., (1978) — Pharmacology of mucus. Brit. Med. Bull. 34 (1) p. 89-94

Parsons, T.S., (1971) — Anatomy of nasal structures from a comparative viewpoint. Handbook of Sens. Physiol. IV Chemical Senses Part 1 ed. Beidler. Springer-Verlag, Berlin. p. 1-26

Phillis, J.W., (1977) — The role of cyclic nucleotides in the CNS. J. Can. Sci. Neurol. 4 (3) p. 152-195

Plattig, K.H., and Kobal, G., (1978) — Human EOG and olfactory evoked brain potentials: techniques and reliability. Results presented in a lecture given at the 3rd ECRO Congress, Pavia

Posternak, T., (1974) — Cyclic AMP and Cyclic GMP. Ann. Rev. Pharmacol. 14 p. 23-33

Poynder, T.M., (1974,a) — Response of the frog olfactory system to controlled odour stimuli. J. Soc. Cosmetic Chem. 25 p. 3-20

Poynder, T.M., (1974,b) — Self-shunting and the frog EOG. Transduction Mechanisms in Chemo-reception. ed. Poynder, I.R.L., London. p. 241-249

Poynder, T.M., (1978) — Personal communication

Poynder et al, (1978) — Poynder, T.M., Bannister, L.H., Gower, D.B., and Booth, W.D. Recording of electrical activity from mammalian olfactory epithelium in vitro: stimulation with pheromones and other odorants. Abstracts from the 3rd ECRO Congress, Pavia. p.40

Reese, T.S., (1965) — Olfactory cilia in the frog. J. Cell Biol. 25 p. 209-230

Reese, T.S., and Brightman, M.W., (1970) — Olfactory surface and central olfactory connections in some vertebrates. Taste and Smell in Vertebrates, ed. Wolstenholme and Knight, J. & A. Churchill London. p. 115-149

Revial et al, (1978,a) — Revial, M.F., Duchamp, A., and Holley, A. Odour discrimination by frog olfactory receptors: a second study. Chem. Sens. and Flav. 3 (1) p. 7-22

Revial et al, (1978,b) — Revial, M.F., Duchamp,A., Holley, A., and MacLeod, P. Frog olfaction: odour groups acceptor distribution and receptor categories. Chem. Sens. and Flav. 3 (1) p. 23-34

Schafer, R., and Brower, K.R., (1975) — Psychophysical recognition of functional groups on odorant molecules. Olfaction and Taste V, ed. Denton and Coghlan, Academic Press,Inc., New York. p. 313-316

Seeman, P., (1972) — The membrane actions of anaesthetics and tranquilisers, Pharmacol. Rev. 24 p. 583-655

Shibuya, T., (1960) — The electrical response of the olfactory epithelium of some fishes. Jap. J. Physiol. 10 p. 417-326

Shibuya, T., (1964) — Dissociation of olfactory neural response and mucosal potential. Science, 143 p. 1338-1340

Shibuya, T., (1969) — Activities of single olfactory receptor cells. Olfaction and Taste III, ed. Pfaffmann, Rockefeller University Press, New York City. p. 109-116

Shibuya, T., and Tonosaki, K., (1972) — Electrical responses of single olfactory receptor cells in some vertebrates. Olfaction and Taste IV ed. Schneider, W.V.G., Stuttgart. p. 102-108

Shibuya, T., and Tucker, D., (1965) — Single unit responses of olfactory receptors in vultures. Olfaction and Taste II ed. Hayashi, Pergamon Press Oxford. p. 219-232

Singer, S,J., (1967) — Covalent labelling of active sites, Advance in Protein Chem. 22 p. 1-54

Singer, S.J., (1970) — Affinity labelling of protein active sites, Molecular Properties of Drug Receptors ed. Porter and O'Conner, J & A Churchill, London p. 229-242

Singer et al, (1973) — Singer, S.J., Ruoho, A., Kieffer, H., Lindstrom, J., and Lennox, E.S., The use of affinity labels in the identification of receptors. Drug Receptors, ed. Rang, Macmillan Press, Ltd., p. 183-191

Steinbrecht, R.A., (1969) — Comparative morphology of olfactory receptors. Olfaction and Taste III, ed. Pfaffmann, Rockefeller University Press. p. 3-21

Takagi, S.F., (1967) — Are EOG's generator potentials? Olfaction and Taste II, ed. Hayashi, Pergamon Press, London. p. 167-179

Takagi, S.F., (1969) — EOG problems. Olfaction and Taste III ed. Pfaffmann, Rockefeller University Press, New York. p. 71-91

Takagi, S.F., (1971) — Degeneration and regeneration of the olfactory epithelium. Handbook of Sens. Physiol. IV Chemical Senses 1- Olfaction, ed. Beidler, Springer-Verlag, Berlin. p. 75-94

Takagi, S.F., and Shibuya, T., (1959) — 'On' and 'Off' responses of the olfactory epithelium. Nature, 184 p.60

Takagi, S.F., and Wyse, G.A., (1965) — Ionic mechanisms of olfactory receptor potentials. Proc. XXIII rd. Int. Congr. Physiol. Sci., Tokyo. (Abstract)

Takagi, S.F., and Yajima, T., (1964) — Electrical responses to odours of degenerating olfactory epithelium. Nature 202 p. 1220

Takagi, S.F., and Yajima, T., (1965) — Electrical activity and histological change of the degenerating olfactory epithelium J. Gen. Physiol. 48 p. 559-569

Takagi et al, (1960) — Takagi, S.F., Shibuya, T., Higashino, S. and Arai, T. The stimulative and anaesthetic actions of ether on the olfactory epithelium of the frog and the toad. Jap. J. Physiol. 10 p. 571-584

Takagi et al, (1966) — Takagi, S.F., Wyse, G.A., and Yajima, T. Anion permeability of the olfactory receptor membrane. J. Gen. Physiol. 50 p. 473-489

Takagi et al, (1968) — Takagi, S.F., Wyse, G.A., Kitamura, H., and Ito, K. The roles of sodium and potassium ions in the generation of the electro-olfactogram. J. Gen. Physiol. 51 p. 552-578

Takagi et al, (1969,a) — Takagi, S.F., Kitamura, H., Imai, K., and Takeuchi, H. Further studies on the roles of sodium and potassium in the generation of the electro-olfactogram: effects of mono-, di-, and trivalent cations. J. Gen. Physiol. 53 p. 115-130

Takagi et al, (1969,b) — Takagi, S.F., Aoki, K., Iino, M., and Yajima, T., The electropositive potential in the normal and degenerating olfactory epithelium. Olfaction and Taste III ed. Pfaffmann, Rockefeller Univ. Press., New York City. p. 92-108

Thornhill, R.A., (1967) — The ultrastructure of the olfactory epithelium of the lamprey, Lampetra fluviatilis. J. Cell Science 2 p. 591-602

Thurm, U., (1972) — The generation of receptor potentials in epithelial receptors. Olfaction and Taste IV, ed. Schneider, W.V.G., Stuttgart. p. 95-101

Tucker, D., (1963) — Olfactory, vomeronasal and trigeminal receptor responses to odorants. Olfaction and Taste I, ed. Zotterman, Pergamon Press. p. 45-69

Tucker, D., (1965) — Electrophysiological evidence for olfactory function in birds. Nature 207 p. 34-36

Tucker, D., and Shibuya, T., (1965) — A physiologic and pharmacologic study of olfactory receptors. Cold Spring Harb. Symp. Quant. Biol. Vol. XXX Sensory Receptors. p. 207-215

Tucker et al, (1975) — Tucker, D., Graziadei, P., and Smith, J. Recovery of olfactory function in pigeons after bilateral transection of the olfactory nerves. Olfaction and Taste, V, ed. Denton and Coghlan, Academic Press Inc., New York, p. 369-374

Usukura, J., and Yamada, E., (1978) — Observations on the cytoemma of the olfactory receptor cell in the newt. Cell & Tissue Res. 188 p. 83-98

Vogel, A.I., (1956) — Practical Organic Chemistry, Longmans, London. p. 379-400

Webb, J.L., (1966) — Comparison of halogenated acids and their derivatives..(as alkylating agents). Enzymes and Metabolic Inhibitors 3 p. 270-281

Wofsy et al, (1962) — Wofsy, L., Metzger, H., and Singer, S,J. Affinity labelling: a general method for labelling the active sites of antibody and enzyme molecules. Biochemistry 1 (6) p. 1031-1039

Yamamoto et al, (1965) — Yamamoto, T., Tonosaki, A., Kurosawa,T. Electron microscope studies on the olfactory epithelium in frogs. Acta Anat. Nippon 40 p. 342-353

Zwaardemaker, H., (1895) — Die Physiologie des Geruchs. Leipzig: Engelmann